Molecular Regulators of Stem Cell Fate and Tumor Development in the Cerebellum

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

Medulloblastoma (MB) is a highly malignant brain tumor that occurs primarily in children. Although surgery, radiation and high-dose chemotherapy have led to increased survival, many MB patients still die from their disease, and patients who survive suffer severe long-term side effects as a consequence of treatment. Thus, more effective and less toxic therapies for MB are critically important. Identifying new treatments will require an understanding of early stages of tumor development, as well as the pathways that are critical for the growth and maintenance of established tumors. In these studies, we explore the roles of WNT signaling in cerebellar development and tumor initiation and of Survivin in the proliferation and survival of tumors.

The WNT pathway plays multiple roles in neural development, is crucial for establishment of the embryonic cerebellum, and is highly expressed in a subset of MBs. However, the cell types within the cerebellum that are responsive to WNT signaling remain unknown. We show that expression of activated β-catenin promotes proliferation of cerebellar neural stem cells (NSCs), but interferes with their capacity for self-renewal and differentiation. These studies suggest that the WNT pathway is a potent regulator of cerebellar stem cell growth and differentiation and that NSCs may represent a cell of origin for WNT-associated MB.

In addition to understanding early stages of transformation, identifying vulnerabilities of established tumors will be critical for development of targeted therapies. We have focused our studies on the role of the inhibitor of apoptosis protein (IAP) Survivin in Sonic hedgehog (SHH)-driven MB. Here we show first that Survivin is overexpressed in murine SHH-driven MB. Using genetic and pharmacological

approaches, we demonstrate that inhibition of Survivin impairs proliferation and survival of both murine and human MB cells. These studies highlight the importance of Survivin in SHH-driven MB, and suggest that it may represent a novel therapeutic target in patients with this disease.

Dedication

I would like to dedicate this dissertation to my Gaka. You always had such faith in me and you were so excited to see me become Dr. Brun #3 in the family. Without your love and support, I could not have accomplished this. I miss you and love you always.

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Last but not least, I want to thank Gaka. You are the most amazing person I have ever known and I was so privileged to get to grow up with you. You always told me how proud you were that I was going to be the third Dr. Brun and your encouragement kept me working hard to accomplish my goals. I will cherish our summers at camp together forever and I can only hope to emulate your warmth, your humor, and caring spirit.

List of Abbreviations

BBB- blood-brain barrier

BIR- baculovirus IAP repeat

BrdU- bromodeoxyuridine

BTB- blood-tumor barrier

CED- convection enhanced delivery

CPC- chromosomal passenger complex

CT- Computerized tomography (scan)

DAPI- 4',6-diamidino-2-phenylindole

E13.5- embryonic day 13.5

EGL- external granule layer

FBS- fetal bovine serum

FUS- focused ultrasound

G-APC- hGFAP-Cre; $Apc^{lox/lox}$ mice

G-Cat- hGFAP-Cre; $Catnb^{lox(Ex3)}$ mice

GEMMs- genetically engineered mouse models

GFR- growth factor reduced (matrigel)

GNPs- granule neuron precursors

IAP- inhibitor of apoptosis protein

IGL- internal granule layer

IsO- isthmus organizer

IT- intratumoral injection

i.p.- intraperitoneal injection

MB- medulloblastoma

MBEN- medulloblastoma with extensive nodularity

M-Cat- Math1-Cre; $Catnb^{lox(Ex3)}$ mice

MCSP- Math1Cre;$Survivin^{fl/fl}$;$Ptch^{fl/fl}$ mice

MDT- multidrug resistance transporters

MR- magnetic resonance (imaging)

Nu/Nu- nude mice, immunocompromised

NSCs- neural stem cells

NSG- NOD;SCID;Gamma mice, immunocompromised

PAMPA-parallel artificial membrane permeability assay

PBS- phosphate buffered saline

PD- pharmacodynamics

PDL- poly D lysine

PDXs- patient derived xenografts

PFA- paraformaldehyde

PI- propidium iodide

PK- pharmacokinetics

P7- postnatal day 7

qRT-PCR- real time quantitative polymerase chain reaction

SBMRI- Sanford Burnham Medical Research Institute

SHH- sonic hedgehog

SP-$Survivin^{fl/fl}$;$Ptch^{+/-}$ mice

TPCs- tumor propagating cells

VZ-ventricular zone

WHO- World Health Organization

WT- wild type

3H-Td- [methyl]^3 Thymidine

7AAD- 7-Aminoactinomycin

1. Introduction

1.1 Medulloblastoma

Medulloblastoma (MB) is an embryonal tumor of the cerebellum and is the most common malignant brain tumor that occurs in children[1]. Although it presents primarily in infants and children, with peak incidence between 3-7 years of age, MB also accounts for 1-2% of adult brain tumors[2]. MBs were originally treated as a single disease, but recognition of the heterogeneity of this disease in terms of histology and pathology, as well as response to treatment, led to stratification into defined histological subtypes. World Health Organization (WHO) designations for the disease have developed over the years as our understanding of the disease has increased and as of 2007, the WHO recognizes five histological subtypes: classic, anaplastic, large cell, desmoplastic/nodular, and medulloblastoma with extensive nodularity (MBEN)[3], although the majority of studies have combined large cell/anaplastic phenotypes. Most MBs display differentiation primarily along the neuronal lineage, with minimal display of glial markers[4]. Classical MBs, which compromise ~65% of cases, consist of sheets of undifferentiated small, round cells with characteristic high cytoplasmic-to-nuclear ratio. Anaplastic/large cell tumors are more aggressive tumors, characterized by pleomorphic cells and high mitotic indexes compared to other types of MB. Desmoplastic/nodular MBs contain areas of more differentiated, non-proliferative cells (nodules) that are reticulin negative surrounded by areas of desmoplasia. MBEN tumors display larger and more frequent nodules than desmoplastic/nodular tumors[4]. Prognosis for MBEN and desmoplastic/nodular patients is more favorable than for those with classical MB[5], while large cell/anaplastic tumors are associated with display the worst prognosis[6].

Early studies of familial inherited mutations that predisposed to MB development provided the first insights into the different molecular drivers of the disease. Germline mutations in PTCH1,a negative regulator of the sonic hedgehog (SHH) pathway, are associated with Gorlin syndrome, which is characterized by a high occurrence of spontaneous MBs [7,8]. Similarly, patients with germline mutations in the SHH pathway component SUFU are predisposed to development of MB[9]. These cases highlight the importance of SHH signaling for driving MB in patients. Turcot's syndrome, a disorder characterized by increased incidence of colon cancer and brain tumors (often medulloblastomas) results from germline mutations in the adenomatous polyposis coli (APC) gene, a negative regulator of canonical WNT signaling[10]. In addition, 15-20% of sporadic medulloblastomas harbor activating mutations in β- catenin or inactivating mutations in APC[11-14]. These and other studies have led to the understanding that many the pathways that are dysregulated in MB (such as SHH and WNT) are critical for normal cerebellar development.

In the past ~6 years, there is has been a huge outpouring of data regarding the molecular alterations that occur in MBs. Consensus from numerous genetic and genomic studies has solidified the classification of MBs into four main subtypes-SHH, WNT, Group 3, and Group 4- with the possibility of further subgroups within these types[15]. Importantly, these molecular subgroups of MB display distinct genetic and molecular alterations, clinical features, and prognosis[15-19]. The best studied of these are the SHH subtype, so designated because tumors are associated with mutations or activation of the SHH signaling pathway, which represent ~28% of human MBs. Interestingly, these tumors have a bimodal age distribution, with the majority occurring

in infants or in teenagers and adults, but few in children[16,19]. SHH driven tumors commonly display classic or desmoplastic histology, do not frequently metastasize, and are associated with intermediate survival[15]. The WNT subtype is least frequent subtype, accounting for about 10-11% of MB cases[16,17]. These tumors have the most favorable prognosis, with recent estimates that >90%of patients survive their disease[15], although they still suffer from debilitating side effects of the therapies. In fact, these patients are likely over-treated and current clinical trials are being developed to address scaling back radiation for these patients to minimize toxicities[15]. These tumors typically occur in children above the age of three, commonly display classical histology, and rarely metastasize[16,17]. These two subtypes will be the focus of the studies presented in Chapters 2 and 3. Group 3 and Group 4 MB are less well characterized and display worse prognosis than the other two subtypes. These tumors are more metastatic and often display large cell/anaplastic phenotypes[16,19].

The identification of medulloblastoma subgroups has not yet influenced clinical practice to any large degree. Patients are still designated as high or low risk based on age at diagnosis, residual tumor size, and presence of leptomenigial metastasis alone[17]. Genomic stratification of patients and molecularly targeted therapies are just starting to be implemented in clinical trials for SHH driven MB[20,21], but are not yet common practice. The current standard of care for MB (independent of subtype) is surgical resection, followed by cranio-spinal radiation (for children above 3 years of age) and high dose chemotherapy. For patients with non-disseminated disease, upwards of 80% are expected to survive beyond 5 years post treatment with the current aggressive treatment regimen[22]. Unfortunately, these treatments are associated with long term consequences affecting quality of life, especially in young children. Severe cognitive

deficits, endocrine disorders, and increased incidence of other cancers later in life occur in many MB survivors[23]. Moreover, still almost a third of the patients remains incurable and will eventually succumb to the disease. Therefore, to minimize detrimental aspects of treatment and to continue improving overall cure rates in patients, more targeted, specific therapies are needed. These therapies may need to be subtype specific, and identification of appropriate strategies will require a more in depth understanding of the early steps of neoplastic transformation and identification of pathways that tumors rely on for growth and maintenance.

1.2 Cell of origin

Cancer develops from progeny of normal cells that acquire somatic mutations that allow them to survive and proliferate unchecked. The “cell of origin” is a term that describes the normal cells that acquire these initial driver mutations and subsequently give rise to tumors. Understanding these normal-tumor cell relationships is crucial for understanding cancer progression and identifying vulnerabilities for novel therapies. Different populations of cells are responsive to particular signaling pathways during normal development, which orchestrate their growth, proliferation, migration, and differentiation. These pathways may be co-opted in cancer, causing transformation in susceptible cell types, which can provide insights into early drivers of the disease. There is clearly a relationship between normal cerebellar development and drivers of MB tumorigenesis, as two of the major subtypes are characterized by activation of cerebellar developmental pathways (SHH and WNT)[15]. Importantly, these subgroups maintain distinct signatures, biological behaviors (where tumors are located, how they develop), and clinical prognosis[16,19,24], supporting the notion that it is important to understand the

underlying basis of these differences. The distinct subtypes of medulloblastoma are likely due at least in part to the different cellular contexts in which these tumors arise, and these differences may be responsible for their differential responses to therapy[25].

Importantly, identification of the cell of origin for the subtypes of MB allows for the development of mouse models that phenocopy the human disease. These models provide insights into the early stages of transformation and can be used to identify critical genetic /epigenetic alterations that occur during tumor development and progression. This will allow for identification of particular vulnerabilities of each subtype of the disease that can be targeted for therapies. These models also provide an important preclinical platform for testing of novel therapeutics. It is therefore of great interest for both researchers and clinicians to identify and characterize the cell of origin for each of the MB subtypes.

In order to explore the cell of origin, we first need to examine the populations of normal cells in the cerebellum that could potentially give rise to tumors and the signals that regulate their development.

1.2.1 Normal Cells of the cerebellum

There are two main germinal zones in the developing cerebellum that produce the majority of cell types: the ventricular zone (VZ) and the rhombic lip. Most cells types in the cerebellum are derived from the VZ; it contains multipotent stem cells that give rise to Purkinje, stellate, basket and golgi interneurons[26,27]. Granule neuron precursors (GNPs), in contrast, are derived from the rhombic lip[28], and give rise to a single cell type, the granule neuron[27]. These germinal zones for stem cells and granule neuron precursors are thought to be the origins for different subtypes of MB.

The neuroepithelial VZ is located in the roof of the fourth ventricle and consists mainly of multipotent cerebellar neural stem cells (NSCs)[27,29]. These cells proliferate in the VZ during embryogenesis, then migrate toward the surface of the cerebellum and differentiate to form the majority of glia and neurons. The first cell type they give rise to is the nuclear neurons (E10-12) which migrate into the deep layers of the cerebellum. The next major cell type, the Purkinje cells, are born and exit the VZ from E11-13, migrate radially into the cerebellar anlage, and eventually form a single cell layer suspended below the molecular layer and above the future internal granule layer. Around the time Purkinje cells stop proliferating, a small number of stem cells from the VZ migrate to the rhombic lip, turn on the transcription factor Atoh1/Math1 (committing to the granule lineage) and generate GNPs. As development continues, the VZ diminishes and gives rise to golgi neurons, and stellate and basket cells that populate the molecular layer postnatally[30]. The VZ eventually disappears during postnatal development. In addition to these stem cells, there are a small number of stem cells that reside in the white matter of the postnatal cerebellum[31]. These cells are characterized by expression of the cell surface marker Prominin1/CD133 and lack of glial and neuronal lineage markers. It is unclear what their contribution to developing cerebellum is, although they are capable of self-renewal and differentiation into neurons, oligodendrocytes, and glia *in vitro* and following transplantation.

GNPs are lineage restricted progenitors that are born in the rhombic lip, proliferate, and begin to migrate tangentially across the surface of the cerebellum around E13 to form the external granule layer (EGL) (below the meninges and above the molecular layer)[27,29,32]. These cells are characterized by expression of Math1 and Zic1, among other factors. Cells in the outer EGL proliferate extensively in response to SHH

ligand produced by neighboring Purkinje cells[33] and form a thick layer, peaking in size at postnatal day 7. As cells migrate from the outer to inner EGL, they start to lose Math1 expression and exit the cell cycle. Inner EGL cells then migrate along Bergmann glial fibers through the molecular layer and past the Purkinje cell layer, and differentiate into mature granule neurons, forming the internal granule layer. By P21, all GNPs have migrated inward, leaving the surface of the cerebellum free of cells. In humans, the GNPs appear in the EGL by week 27 and the differentiation/migration process that clears the surface of the cerebellum continues through the first year of life[29]. A very recent study has identified another small population of EGL resident cells that are distinct from GNPs and also give rise to granule neurons[34]. These cells are characterized by Nestin expression, are relatively quiescent, and do not proliferate in response to SHH *in vivo.*

1.2.2 Signals that regulate cerebellar development

Early stages of development of the cerebellar primordium are orchestrated by signals from the isthmus organizing center[32]. The isthmus organizer (IsO) region occurs in the neural tube at the boundary between what will be the midbrain and hindbrain, and importantly defines the anterior limit of the cerebellum through expression of FGF8. The position of the IsO is dependent on the reciprocal repression of two key molecules, Otx2 and Gbx1, which are expressed in the mid- and hindbrain respectively[35]. Mistargeting experiments have highlighted the importance of each for modulating the positioning of the IsO and establishment of a sharp boundary[36,37]. The IsO, in turn, expresses and secretes key regulators of cerebellar development such as FGF8, WNT1, Engrailed 1 and 2, and Pax 2 and 5[38], all of which feedback on each other to ensure correct patterning of the cerebellum. Inactivation or deletion of each of these molecules in

animals causes large deletions or complete ablation of the cerebellum [39-41,32,42], suggesting that they represent important regulators of early cerebellar development. Additionally, expression of Hox2A has been implicated in defining the posterior boundary of the cerebellum, as mice without Hox2A develop much larger cerebella[43].

There are a myriad of factors that contribute to the highly coordinated migration, proliferation, and eventual mitotic exit and differentiation of GNPs. Signaling for the specification of granule cells in the rhombic lip is initiated by bone morphogenic proteins (TGFbeta family proteins), specifically BMP6, BMP7 and Gdf7[44] that are secreted from cells in the roof plate of the fourth ventricle[44]. These molecules stimulate Math1 expression. Loss of Math1 (Math1-/- mice) causes complete abrogation of the EGL and loss of granule neurons in the cerebellum[45], suggesting that expression of this transcription factor is critical for GNP development. Once the cells reach the EGL, the predominant mitogenic signal for GNPs is SHH ligand, which is secreted by neighboring Purkinje cells[33]. Mutation or loss of Purkinje cells results in decreased proliferation and lower cell number of GNPs[46,47], suggesting that signaling from these cells is crucial for normal GNP proliferation and expansion. Further, GNPs express the receptor for the SHH ligand, PTCH, and display increased expression of SHH target genes such as CyclinD2 and Nmyc, both of which are essential for GNP proliferation[48,49]. Activation of Notch signaling in GNPs (which express Notch2), suggests that this pathway can promote proliferation as well[50]. Other factors with known roles in GNP proliferation are Ru49/Zipro1, Zic1, and Zic2[51-53], all of which are expressed in the EGL.

As cells move to the inner EGL, they begin to exit the cell cycle. Signals that control this decision to become postmitotic and to migrate and differentiate are not well understood. Importantly, GNPs lose Math1 expression and increase NeuroD

expression, which is critical for GNP survival during differentiation and migration[54]. Loss of CXCR4, a receptor expressed in GNPs, or of its ligand CXCL12/SDF1-α, causes aberrant migration of a subset of GNPs into the IGL prior to differentiation, suggesting that signaling through CXCR4 is critical for maintenance of GNPs in the EGL[55,56]. Additionally, inner EGL GNPs upregulate Eng2, which promotes cell cycle exit and differentiation of GNPs[57]. It has been suggested that suppression of SHH signaling may contribute to the switch from proliferation to differentiation. Molecules such as bFGF and BMP2 (through SMAD5) are capable of antagonizing the proliferative response of GNPs to SHH and inducing differentiation[58,59] , and have been suggested to play a role in this process. Notch2 is downregulated as GNPs exit the cell cycle and differentiate as well[50], so loss of Notch signaling could contribute to the decreased proliferation and differentiation of these cells[50]. Additionally, p27/Kip1 (cyclin dependent kinase inhibitor) expression is limited to cells residing in the inner EGL[60] and may contribute to the post-mitotic state of these cells.

In addition to proliferation, a low basal level of controlled apoptosis characterizes the EGL and contributes to controlling GNP cell number. Brain-derived neurotrophic factor (BDNF) and insulin-like growth factor (IGF1) signaling have been implicated as mediators of cell survival for this cell population[61,62]. Additionally, mutations in GIRK2, an inward rectifying K+ ion channel, have demonstrated the requirement of this protein for cell survival during differentiation (to counteract depolarization signaled by NMDA receptor activation during differentiation)[63,64] .

One of the major signaling pathways that has been identified for the terminal migration of GNPS is Netrin and its receptor Unc5H3, a pathway known to regulate neuronal migration and axonal projection in the CNS[65]. Unc5H3 is expressed on GNPs

and when mutated, cerebellar cells aberrantly migrate outside the normal boundaries of the cerebellum[66]. Similarly, Pax6 (Sey) mutant mice display abnormal migration, with post mitotic GNPs scattered through the EGL suggesting that Pax6 is another regulator of correct GNP migration[67]. This phenotype may be mediated in part by downregulation of Unc5H3 expression in the Pax6 mutant mice. Finally, interference with the interaction of GNPs with glial cells during migration by disrupting binding of Neuregulin to glial Erb4 or blocking/mutating astrotactin prevents normal migration as well[29].

Less is known about the signals that regulate the stem cell populations in the cerebellum. It is known that these cells can proliferate in response to bFGF in vitro[31], in contrast to GNPs that differentiate in the presence of bFGF[58]. The Notch pathway is active in the VZ as well: deletion of Notch1 in early cerebellar cells (using Eng2-Cre) causes failure to differentiate into cells along the neural lineage in the cerebellum and increases apoptosis, pointing to a critical role of Notch signaling in determining fate of early cerebellar stem cells[68]. A recent study has demonstrated that expression of Ptf1a, a pancreatic transcription factor, in VZ cells is essential for their ability to produce Purkinje cells and interneurons[69], and loss of Pfta1 allows some VZ derived cells to switch to an EGL progenitor cell fate[70]. So signaling through this pathway may be critical for VZ stem cell fate determination as well. The WNT pathway is clearly a critical regulator of early cerebellar development, as evidenced by complete ablation of the cerebellum in WNT1 knockout mice[71]. It is likely therefore that early stem cells may respond to WNT signaling. Additionally, WNT signaling is known to regulate progenitor and stem cell proliferation in the colon and CNS[72,73]. But its role in later stages of cerebellar development, and the identity of the cells in cerebellum that respond to WNT signaling, remain unclear. Deletion of β-catenin in Nestin+ cells prevents formation of the

cerebellar vermis without grossly affecting neuronal precursor diversification[74]. Additionally, activating and loss of function studies have suggested a role for WNT signaling in foliation of the cerebellum and in differentiation fate of cells born from the cerebellar ventricular zone[75,76]. We will address the role of WNT signaling in cerebellar progenitors more specifically in Chapter 2.

1.2.3 Potential Cells of origin for medulloblastoma subtypes

The best studied subtype of medulloblastoma is the SHH subtype, and numerous mouse models of this subtype have been developed. Using a temporally controlled conditional deletion of the PTCH receptor, Yang et al have demonstrated that these tumors arise from the cerebellar granule cell lineage[34]. This was long suspected, as the SHH tumors share many characteristics with GNPs, including expression of the transcription factor Math1 and activation of SHH target genes. Further, pre-neoplastic lesions in *Ptch* mutant mice reside in the EGL and are capable of either differentiating into granule neurons or progressing to MB in a subset of mice[77,78]. Recent studies have demonstrated that other cells can also serve as cells of origin for this subtype, including stem cells[79] and Nestin+ EGL cells[34], although stem cells must acquire characteristics of the GNP lineage for these tumors to form[79].

In terms of the WNT-driven subtype of MB, work from the Gilbertson lab has demonstrated that these tumors can actually arise from cells outside the cerebellum[24]. BLBP+ cells from the floor of the fourth ventricle (dorsal brain stem progenitors) can be transformed by activation of β-catenin and loss of p53 into tumors in ~15% of mice . These tumors resemble human WNT tumors in terms of location in the brain and gene expression data. While these studies identify a putative cell of origin for WNT tumors, this may only represent a subset of WNT-driven tumors, namely those with p53

inactivation. It is possible that cells from within the cerebellum could also give rise to tumors. Our studies in chapter 2 examine the effects of activated WNT signaling in cerebellar progenitors and demonstrate that WNT signaling is mitogenic for cerebellar stem cells, but not for GNPs.

The other two subgroups of medulloblastoma are not as well defined or studied. Currently no model exists for Group 4 tumors. In Group 3 tumors, one of the major alterations identified in this group is amplification or overexpression of the c-myc oncogene. Very recently, Dr. Yanxin Pei from our lab developed first model of this disease, an orthotopic allograft model derived from viral overexpression of c-myc and dominant negative p53 in postnatal cerebellar stem cells[80]. Importantly, this model recapitulates many features of the human disease. These studies came out concurrently with work by Kawauchi et al. showing that overexpression of c-myc in p53-null GNPs allows these cells to form Group 3-like tumors following transplantation[81]. Interestingly, both groups demonstrated that tumors generated from Atoh1+ GNPs lose their neuronal lineages markers and more closely resemble neural stem cells or induced pluripotent cells by expression profiling. This suggests that for Group 3 tumors, these oncogenes have to be activated either in a stem cell, or the progenitor cell has to acquire a stem-like phenotype (possibly through de-differentiation). So it is likely that stem cells represent the cell of origin for Group 3 tumors.

These data demonstrate how critical developmental signaling pathways interact with particular stem and progenitor cell populations in order to form cellularly and molecularly distinct tumors.

1.3 Molecular targets in established tumors

In addition to studying the early stages of tumorigenesis, examination of established tumors to identify pathways that tumors are dependent on for growth and survival will be critical for defining novel therapies. Genes that are highly expressed in tumors and mutations that commonly occur in these tumors provide insight into vulnerabilities that can be exploited for therapy.

This strategy has been used to identify targeted therapies in numerous cancers. For example, it was discovered in 2002 that 40-50% of melanoma patients have activating mutations in BRAF [82] and as a consequence, exhibit constitutive MAPK pathway activation leading to uncontrolled cell proliferation and evasion of apoptosis[83]. This mutation was also associated with decreased survival in patients with metastastic melanoma[84,85]. The importance of BRAF for tumor growth led to the development of BRAF and MEK inhibitors to treat BRAF dependent melanomas, which both displayed positive responses in clinical trials[86]. Similarly, the recognition that a subset of breast cancers have amplified Her2 (a member of the epidermal growth factor receptor family of tyrosine kinases), and that expression predicts poor outcome in patients, led to testing of anti-Her2 antibodies (e.g. trastuzumab) and tyrosine kinase inhibitors (e.g. lapatinib) in this subset of patients[87]. These and other targeted therapies have shown great results in preclinical models and in trials[86,87], although a common problem is development of resistance.

This approach has recently begun to be utilized in MB as well. Genetic and genomic studies have identified mutations and genomic alterations that are specific for each subtype of MB and that could contribute to tumorigenesis. For example, c-myc amplification or overexpression occurs in Group 3 tumors and mutations in components

of the WNT and SHH pathways occur specifically in their respective subgroups[15]. Recently, epigenetic modifiers have come up as a class of molecules that are commonly altered in MB and there are distinct mutations that occur specifically in Group 3 and 4 (e.g KDM6A, EZH2, MLL2) or in WNT tumors (SMARC4, CREBBP)[17,88,89]. Initial studies in our lab have shown that histone deacetylase (HDAC) inhibitors potently inhibit growth of murine and human Group 3 MB (Yanxin Pei et. al, in review). In the SHH subtype of MB, a number of Smoothened (a mediator of SHH signaling) antagonists (GDC-049, LDE-225, IPI-926, PF5274857) have been developed to target this tumor[90,91]. These compounds have shown promising results in a number of SHH-driven mouse models[90,92-94] and have been moved forward to clinical trials. In one patient with metastatic MB, treatment with GDC-049 caused a spectacular initial response with regression of all tumors[20]. Unfortunately, this response was not long-lived and tumors recurred. This rapid development of resistance is similar to what has been observed with other targeted therapies[95,96]. So even in SHH-associated MB, additional therapeutics will be required to fully eradicate the disease. In Chapter 3, we identify Survivin as a gene of interest in SHH driven MB and investigate the potential of Survivin antagonists as novel therapeutic agents for treatment of MB.

2. WNT signaling increases proliferation and impairs differentiation of stem cells in the developing cerebellum

2.1 Introduction

WNT proteins play crucial roles in nervous system development[97,98]. Mutations in *Wnt1* cause severe defects in the midbrain, hindbrain and developing spinal cord[40,99,100] and ablation of *Wnt3a* results in loss of the hippocampus[101]. Conversely, ectopic expression of β-catenin (a key activator of the canonical WNT signaling pathway) in neural precursors leads to expansion of the progenitor pool and enlargement of the forebrain and spinal cord[102-104]. In addition to its mitogenic effects in some parts of the CNS[105,106] WNT signaling can also regulate cell fate determination[107], differentiation[108], axon growth[109], synapse formation[110,111] and myelination[112]. Thus, WNTs may have distinct effects on different cell types in the developing nervous system.

Among the most widely studied functions of WNT signaling is in the establishment of the midbrain-hindbrain boundary that gives rise to the cerebellum[40]. However, the role of the pathway at later stages of cerebellar development is less well understood. At postnatal stages, Wnt7a is required for axonal branching by granule neurons and facilitates their formation of synapses with mossy fibers[110,113] In addition, recent studies have shown that deletion of β- catenin in nestin-expressing progenitors results in premature neuronal differentiation and hypoplasia of the cerebellar vermis, suggesting that WNT signaling might regulate growth and differentiation in the embryonic and early postnatal cerebellum[74]. Perhaps the most striking evidence for the importance of WNT signaling in the cerebellum is the association between WNT pathway mutations and the cerebellar tumor medulloblastoma. Germline mutations in the

adenomatous polyposis coli (*APC*) gene, a negative regulator of canonical WNT signaling[10], result in Turcot's syndrome, a disorder characterized by increased incidence of colon cancer and brain tumors (often medulloblastomas). In addition, 15-20% of sporadic medulloblastomas harbor activating mutations in β- catenin or inactivating mutations in *APC*[11-14]. Recent studies suggest that WNT-associated medulloblastomas can arise from cells outside the cerebellum, in the dorsal hindbrain[24], however, it remains possible that some of these tumors originate from progenitors within the cerebellum. Understanding the cells from which these tumors arise will be critical for identifying cooperating pathways and potential targets for therapy of this subtype. The ability of progenitors in the cerebellum to proliferate in response to WNT signaling remains poorly studied.

The cerebellum contains two distinct germinal zones: the ventricular zone (VZ), which contains multipotent neural stem cells (NSCs) that give rise to the majority of cerebellar neurons and glia, and the external granule layer (EGL), which contains granule neuron precursors (GNPs) that give rise to a single cell type, the granule neuron[27,29]. To determine which of these cells is susceptible to the mitogenic effects of WNT signaling, we isolated NSCs and GNPs and tested their ability to proliferate following infection with β- catenin-encoding retroviruses. In addition, we used transgenic mice carrying a Cre-inducible allele of β-catenin to examine the effects of activating the WNT pathway in stem cells and progenitors *in vivo*. Our studies reveal that WNT signaling is not mitogenic for GNPs. By contrast, activation of the WNT pathway does promote proliferation of NSCs in the VZ, and these cells undergo expansion during embryonic development. However, this expansion is accompanied by loss of the ability to undergo self-renewal or differentiation and by failure to form most differentiated cell

types in the cerebellum. These studies suggest that WNT signaling plays an important role in regulating the growth and differentiation of stem cells in the developing cerebellum.

These studies were performed in close collaboration with a post doc in the lab Dr. Yanxin Pei and data from each contributor will be indicated in figure legends (YP or SNB).

2.2 Results

2.2.1 Identification of cells in the developing cerebellum that proliferate in response to WNT signaling

Although previous studies have shown that WNT signaling can promote the proliferation of progenitors in the cortex and spinal cord[102,104], mitogenic effects of WNT signaling have not been described in the cerebellum. To determine whether cerebellar progenitors are responsive to canonical WNT signaling, we isolated cells from the embryonic cerebellum and tested their ability to proliferate following infection with retroviruses encoding activated β-catenin. Cells from embryonic day (E) 13.5 or E17.5 were infected with control (GFP) or β-catenin-IRES-GFP viruses for 48 hours, pulsed with tritiated thymidine and assayed for thymidine incorporation.

Whereas cells from E13.5 cerebellum exhibited a 2.5-fold increase in proliferation following infection with β-catenin-encoding cells viruses, cells from E17.5 showed no response (Fig. 1A). E13.5 cells also proliferated in response to soluble Wnt1 protein (Fig. 2). These studies suggested that WNT signaling is mitogenic for cells in the early embryonic cerebellum.

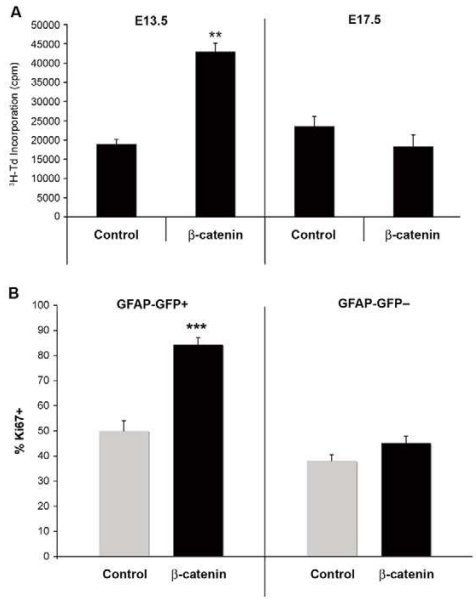

Figure 1:β-catenin increases the proliferation of a subpopulation of cells in the developing cerebellum.

(A)Cells from the cerebellum of E13.5 or E17.5 wild-type (WT) mice were infected with control (GFP only) or β-catenin-IRES-GFP retroviruses for 48 hours, pulsed with tritiated thymidine (3H-Td) and cultured overnight before being assayed for 3H-Td incorporation. Data represent the mean (±s.e.m.) of triplicate samples. (B)GFP+ or GFP– cells were FACS sorted from cerebella of E14.5 hGFAP-GFP transgenic mice and infected with control (YFP only) or β-catenin-IRES-YFP retroviruses for 48 hours. There was no significant difference in infection rates of GFAP+ and GFAP– cells (45% and 40%, respectively). Cells were stained with Ki67 antibodies and the percentage of Ki67+ cells was counted. Data represent the mean (±s.e.m.) percentage of Ki67+ cells in six fields **, *P*<0.01; *, *P*<0.0001; Student's *t*-test. YX.**

To determine which cells in the embryonic cerebellum proliferate in response to WNT signaling, we fractionated cells prior to infection with β -catenin-encoding viruses. The human *GFAP* (hGFAP) promoter is expressed in the VZ of the embryonic cerebellum, and FACS-sorted hGFAP-GFP+ cells can generate neurospheres and differentiate into neurons, astrocytes and oligodendrocytes[79,114,115]. Thus, the hGFAP promoter marks stem cells in the embryonic cerebellum. To determine whether these

cells can proliferate in response to WNT signaling, we FACS sorted GFP+ and GFP– cells from the cerebellum of hGFAP-GFP mice[116] and infected them with retroviruses

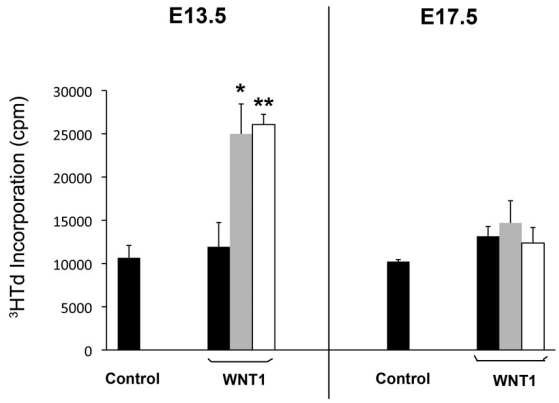

Figure 2:WNT1 protein increases proliferation of cells in the embryonic cerebellum.

Cells were isolated from E13.5 or E17.5 WT cerebellum and cultured for 48 hours in serum-free medium containing no factors (control) or recombinant WNT1 protein (black bars, 10 ng/ml; gray bars, 50 ng/ml; white bars, 200 ng/ml). Cells were pulsed with tritiated thymidine (^{3}H-Td) and cultured overnight before being assayed for 3H-Td incorporation. Data represent the mean of triplicate samples ± s.e.m. *, *P*<0.01; **, *P*<0.001. YX.

encoding β-catenin. After 48 hours, we stained cells with antibodies against the proliferation marker Ki67 and counted Ki67+ cells. As shown in Fig. 1B,C, β-catenin significantly increased the proliferation of GFAP+ cells but not of GFAP– cells. These results suggest that WNT responsiveness is associated with a GFAP+ population in the embryonic cerebellum.

We have previously demonstrated that the postnatal cerebellum also contains NSCs[31]. These represent less than 1% of the cells in the cerebellum during the first

postnatal week, but can be identified based on their expression of prominin 1 (CD133)

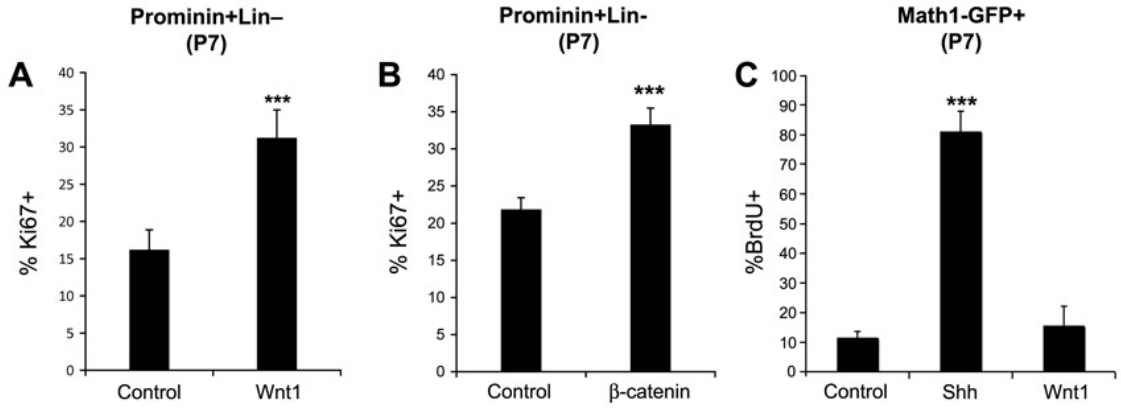

Figure 3:WNT signaling increases the proliferation of postnatal cerebellar stem cells but not granule neuron precursors.

(A,B) Prom1+ Lin− cells were sorted from P7 WT cerebellum and cultured in serum-free medium containing no factors (control) or recombinant WNT1 protein (A) or were infected with control (GFP) or β-catenin-IRES-GFP retroviruses (B). After 3 days, cells were stained with Ki67 antibodies to detect proliferation. (C**) GFP$^+$ cells (GNPs) were sorted from the cerebellum of P7 Math1-GFP mice and cultured in serum-free medium containing no factors (control), SHH or WNT1. After 48 hours, cells were pulsed with BrdU overnight and then stained with anti-BrdU antibodies. Data represent the mean of eight (A), ten (B) or six (C) fields ± s.e.m. ***, *P*<0.001. YX and SNB.**

and their lack of neuronal and glial lineage markers (Prom1+ Lin–). To determine whether postnatal cerebellar NSCs are also responsive to WNT signaling, we sorted Prom1+ Lin– cells from P7 cerebellum and treated them with Wnt1 protein or infected them with β-catenin encoding retroviruses. Both Wnt1 protein and β- catenin increased the proliferation of these cells *in vitro* (Fig. 3A,B). By contrast, GNPs, which represent the majority of cells in the postnatal cerebellum [and express the transcription factor Math1 (Atoh1 – Mouse Genome Informatics)] did not proliferate in response to WNT1 protein or β -catenin encoding retroviruses (Fig 3C; data not shown). Together, these studies suggest that WNT signaling is mitogenic for NSCs in the embryonic and postnatal cerebellum.

2.2.2 Activation of β-catenin expands NSCs in the cerebellar ventricular zone

Having observed that cerebellar NSCs proliferate in response to WNT signaling *in vitro*, we tested their responsiveness to WNT signaling *in vivo*. We used *Catnb*$^{lox(Ex3)}$ mice, in which exon 3 of the β-catenin gene is flanked by loxP sites[104,117]. When this exon is deleted by Cre recombinase, the resulting protein cannot be phosphorylated and degraded and therefore accumulates in the nucleus, where it constitutively turns on expression of WNT target genes. To activate WNT target gene expression in cerebellar NSCs, we crossed *Catnb*$^{lox(Ex3)}$ mice with *hGFAP-Cre* mice[118], which express Cre in the cerebellar VZ[79,114,115]. To verify that β-catenin was activated in the VZ of *hGFAP-Cre; Catnb*$^{lox(Ex3)}$ (G-Cat) mice, we stained sections from E14.5 wild-type (WT) or G-Cat animals with anti- β-catenin antibodies. As shown in Fig. 4A-D, β-catenin was predominantly cytoplasmic in the VZ of WT mice, but was strongly expressed in nuclei of cells within the VZ of mutant mice. These data suggest that G-Cat mice have constitutive activation of the WNT pathway in the cerebellar VZ.

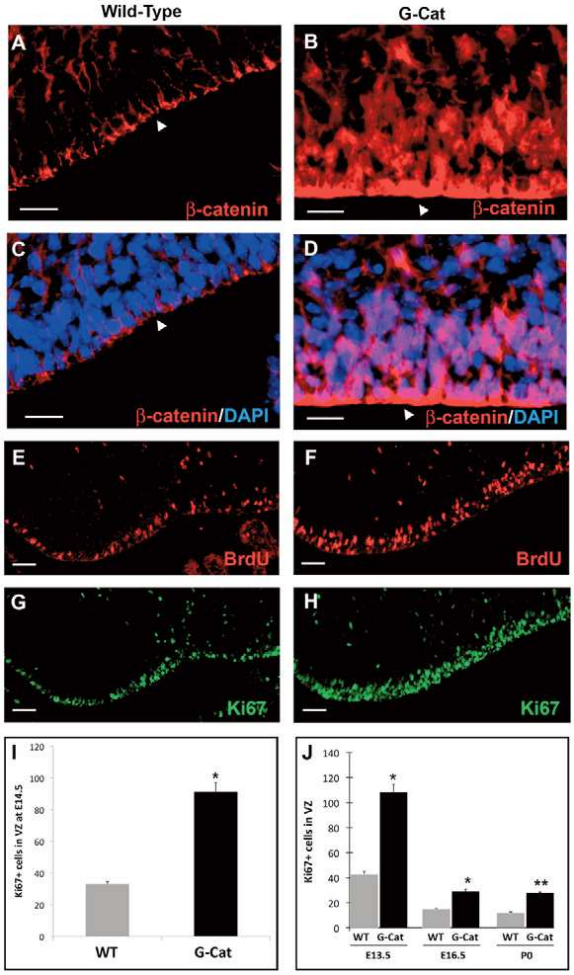

Figure 4:Activated β-catenin promotes proliferation in the cerebellar ventricular zone.

(A,B)β-catenin immunostaining (red) of E14.5 cerebellum reveals increased expression in the ventricular zone (VZ, arrowheads) of *hGFAP-Cre; Catnb$^{lox(Ex3)}$* (G-Cat) mice (B) compared with WT mice (A). (C,D) Colocalization with DAPI (blue) highlights the cytoplasmic localization of β-catenin in WT mice (C) and nuclear localization in G-Cat mice (D). Arrowheads indicate the VZ. (E-H)Staining with anti-BrdU (red, E,F) or anti-Ki67 (green, G,H) shows increased proliferation in the VZ of E14.5 G-Cat mice (F,H) compared with WT mice (E,G). (I,J)Number of Ki67+ cells per section in the VZ of WT and G-Cat mice at E14.5 (I) and at E13.5, E16.5 and P0 (J). Data represent the mean (±s.e.m.) number of Ki67+ cells per section of six sections. *, *P*<0.01; **, *P*<0.001. Scale bars: 50μm. YX.

To assess the effect of WNT signaling on proliferation in the embryonic cerebellum, we examined BrdU incorporation. E14.5 WT and mutant mice were exposed

to BrdU 30 minutes before being sacrificed, and cerebellar sections were stained with anti-BrdU antibodies. As shown in Fig. 4E,F, the VZ of mutant mice contained significantly more BrdU+ cells than that of WT mice (92±8 versus 38±5 cells/section). We also used anti-Ki67 to assess proliferation in WT and mutant cerebella (Fig. 4G-I). At E14.5, we found a 3-fold increase in the proportion of Ki67+ cells in the VZ of mutant mice compared with WT mice, confirming that WNT signaling increases proliferation in the embryonic VZ (Fig. 4I). Constitutively active β-catenin also caused increased proliferation at E13.5, E16.5 and P0, although the effects were most pronounced at earlier stages (Fig. 4J). These data suggest that activated β-catenin promotes proliferation in the cerebellar VZ during embryonic development.

Previous studies have suggested that GFAP-expressing cells in the embryonic cerebellum include multipotent NSCs as well as mature astrocytic cells[79,114,115]. To determine whether the proliferating cells in the VZ of G-Cat mice were NSCs, we stained sections from E14.5 WT and mutant mice with antibodies specific for GFAP and the NSC marker Sox1. Mutant animals exhibited increased numbers of GFAP+ and Sox1+ cells in the embryonic cerebellar VZ (Fig. 5AD). We also co-stained sections from these animals with Sox1 and BrdU antibodies, and observed that almost all of the BrdU+ cells in the VZ expressed Sox1 (Fig. 5E,F). Similar results were obtained using antibodies specific for Sox2 (data not shown), supporting the notion that the proliferating cells in the VZ were NSCs.

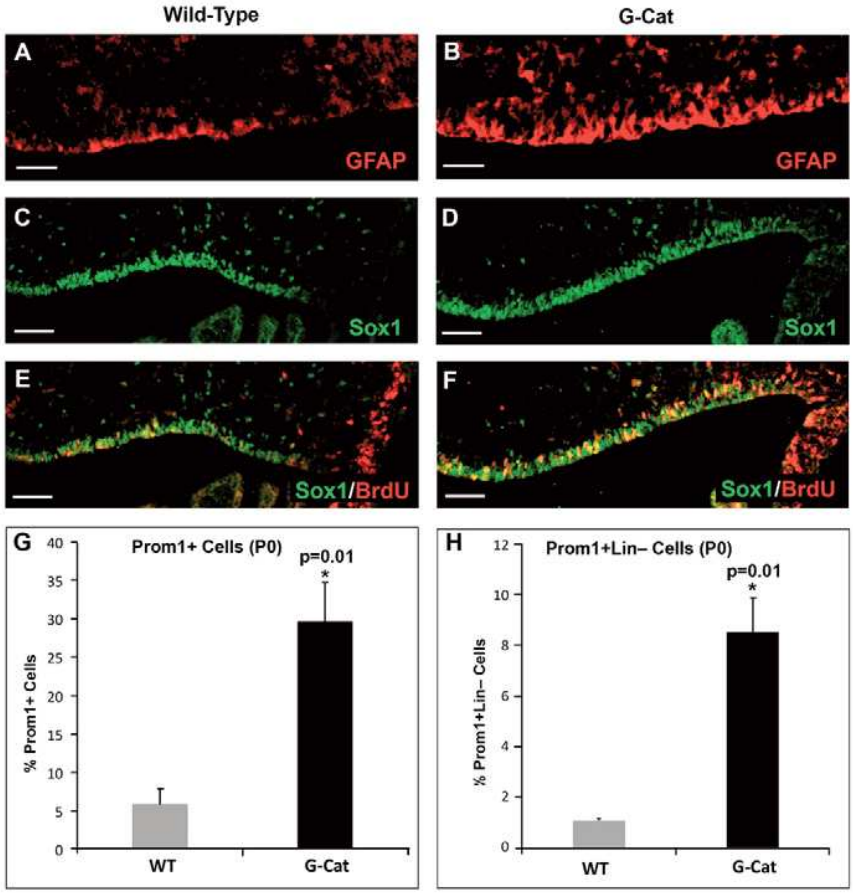

Figure 5: Activated β-catenin increases the number of stem cells in the embryonic and neonatal cerebellum.

(A-F) Cerebellar sections from E14.5 WT (A,C,E) or G-Cat (B,D,F) mice were stained with anti- GFAP antibodies (red in A,B) or anti-Sox1 (green) and anti-BrdU (red) antibodies (C-F). Note the increased expression of GFAP and Sox1 and the colocalization of Sox1 and BrdU in the mutant VZ. (G,H) Increased numbers of progenitors (G) and stem cells (H) in the cerebellum white matter in G-Cat mice compared with WT mice at P0. Cells isolated from the cerebellum of P0 WT and G-Cat mice were stained with antibodies specific for prominin 1 (Prom1) and for lineage (Lin) markers (PSANCAM, O4 and TAPA-1). The percentage of Prom1+ progenitors (G) and Prom1+ Lin– stem cells (H) was analyzed by flow cytometry. Data represent mean (± s.e.m.) percentages from three separate experiments. *, *P*<0.01. Scale bars: 50µm. YX.

To determine whether there was a similar expansion of NSCs in the postnatal cerebellum, we isolated cells from G-Cat mice at P0 and analyzed the percentage of Prom1+ cells (stem cells and progenitors) and Prom1+ Lin– cells (stem cells). Both

populations were increased ~6-fold in mutant compared with WT mice (Fig. 5G,H). Together, these studies suggest that activation of WNT signaling in GFAP+ cells expands the stem cell pool in the embryonic and postnatal cerebellum.

2.2.3 Continued expression of β-catenin disrupts cerebellar development

The fact that β-catenin promoted the proliferation of cerebellar NSCs suggested that it might lead to increased production of neurons and glia and expansion of the cerebellum. Unfortunately, almost all G-Cat mice died shortly after birth, making it impossible to study the long-term consequences of β-catenin activation in these animals. However, even at late embryonic and early postnatal stages the phenotype of mutant mice was not consistent with a purely mitogenic effect of WNT signaling. At E16.5, mutant mice had smaller cerebella than WT mice and, despite expansion of the VZ, exhibited a marked reduction in the thickness of the rhombic lip and EGL (Fig. 6A-D). At P0, mutant mice had an even more severe phenotype: their cerebella were significantly smaller than those of WT mice and lacked the foliation characteristic of this stage of development (Fig. 7A,B). Moreover, staining of the cerebellum with lineage markers revealed marked defects in neuronal and glial differentiation. The region normally occupied by the EGL contained few cells and none of these expressed Ki67 or Pax6, which are markers of proliferating GNPs (Fig. 7A-D). Some calbindin+ Purkinje cells were present, but these were very disorganized compared with those in the WT cerebellum (Fig. 7E,F). Pax2+

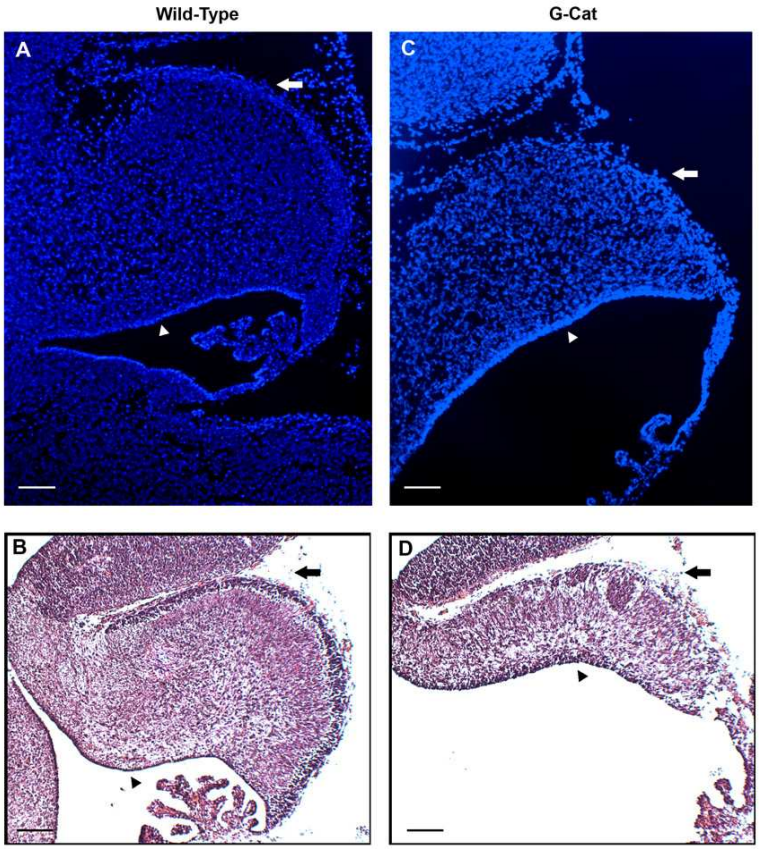

Figure 6:Activation of β-catenin in the ventricular zone disrupts embryonic cerebellar development.

(A-D**) Cerebella of E16.5 WT (A,B) and G-Cat (C,D) mice were sectioned and stained with DAPI (A,C) or Hematoxylin and Eosin (B,D). Although the thickness of the VZ (arrowhead) is increased in the mutant, the overall size of the cerebellum and the thickness of the external granule layer (arrow) are reduced. Scale bars: 50 µm. YX.**

interneuron progenitors (Fig. 7G,H) and glial cells [S100β and BLBP (Fabp7 – Mouse Genome Informatics) staining, Fig. 7I-L] were detectable but in reduced numbers, and, in the case of glia, with aberrant localization and morphology. Thus, the increased NSC proliferation induced by β catenin is accompanied by marked defects in neuronal and glial differentiation.

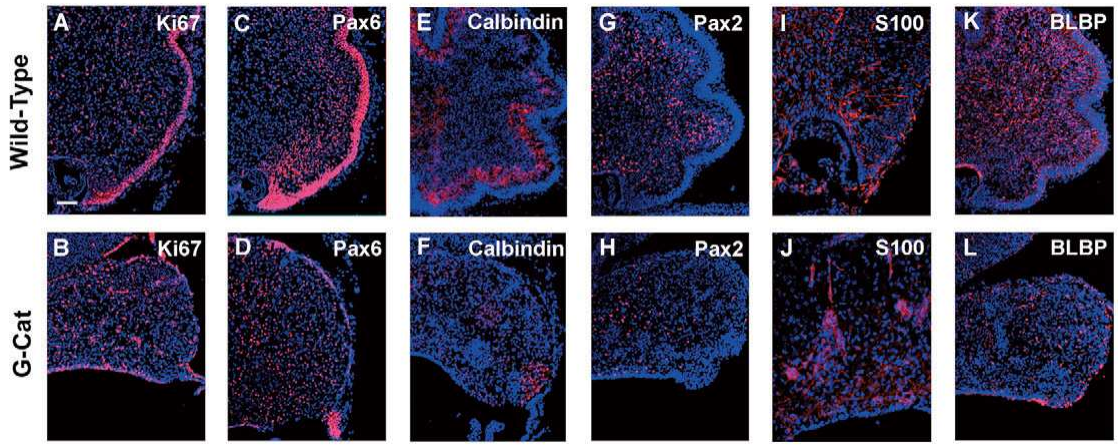

Figure 7:Activation of β-catenin in stem cells impairs neuronal and glial differentiation.

(A-L)Sections from P0 WT (A,C,E,G,I,K) and G-Cat (B,D,F,H,J,L) cerebella were stained with antibodies specific for Ki67 (A,B) to label proliferating cells, Pax6 (C,D) to label granule neuron precursors (GNPs), calbindin (E,F) to mark Purkinje neurons, Pax2 (G,H) to detect stellate and basket interneuron precursors, and S100β (I,J) or BLBP (K,L) to detect glial cells. All sections were counterstained with DAPI. Note the marked reduction in progenitors and differentiated cells in G-Cat mice. Scale bar: 50μm. YX and SNB.

The dramatic loss of neuronal progenitors in G-Cat mice could reflect a failure of NSCs to differentiate along the neuronal lineage or the reduced proliferation or survival of neuronal progenitors once they have been generated. To test whether expression of β-catenin in GNPs impairs their growth or survival, we crossed *Catnb*$^{lox(Ex3)}$ mice with Math1-Cre transgenic mice, which express Cre in cells of the granule lineage[79,115]. Analysis of cerebella from *Math1-Cre; Catnb*$^{lox(Ex3)}$ (M-Cat) mice at E14.5 revealed an intact EGL with relatively normal numbers of proliferating (Ki67+) cells (Fig. 8A,B). At P0, proliferation could be detected in the anterior EGL, but few proliferating cells were seen on the posterior surface of the cerebellum (Fig.8C,D). Although these animals exhibited normal viability and no overt signs of neurological dysfunction, their cerebella were

smaller and contained fewer lobes than those of WT animals (Fig. 8E,F). These results suggest that expression of activated β-catenin does not prevent formation of the EGL, but might impair proliferation or long-term survival of GNPs.

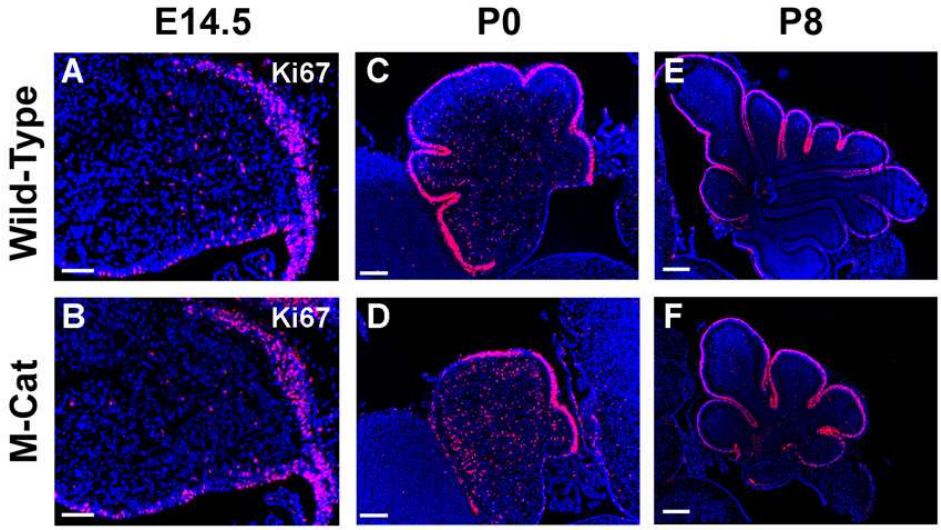

Figure 8: Overexpression of β-catenin in granule neuron precursors reduces cerebellar size and foliation.

(A-F**) Cerebellar sections from WT (A,C,E) and *Math1-Cre; Catnb$^{lox(Ex3)}$*) (M-Cat: B,D,F) mice were stained with Ki67 antibodies to detect proliferation at (A,B) E14.5, (C,D) P0 and (E,F) P8. Scale bars: 50 µm. YX.**

2.2.4 Deletion of Apc impairs the function of cerebellar NSCs

A possible explanation for the defects in G-Cat mice is that the non-degradable (exon 3-lacking) β-catenin protein in these animals leads to high levels of WNT pathway activation, which might be toxic for stem cells and progenitors. To examine this possibility, we used an alternative approach to activating WNT signaling in these cells: inactivation of the Apc protein. Apc is part of the 'destruction complex' that targets β-catenin for degradation in the absence of WNT stimulation; inactivating mutations in Apc

allow for accumulation and nuclear translocation of β-catenin, thereby promoting WNT target gene transcription[119].

To study the consequences of Apc inactivation for cerebellar NSCs we crossed *hGFAP-Cre* mice with *Apc*$^{lox/lox}$ mice, which carry loxP sites surrounding exon 14 of the *Apc* gene. This exon is crucial for interaction of Apc with β-catenin and axin (another component of the destruction complex), so following Cre-mediated deletion Apc is unable to target β-catenin for degradation. To verify that this approach resulted in WNT pathway activation, we stained cerebella from E14.5 *hGFAP-Cre; Apc*$^{lox/lox}$ (G-APC) mice with anti- β-catenin. Deletion of *Apc* exon 14 resulted in nuclear accumulation of

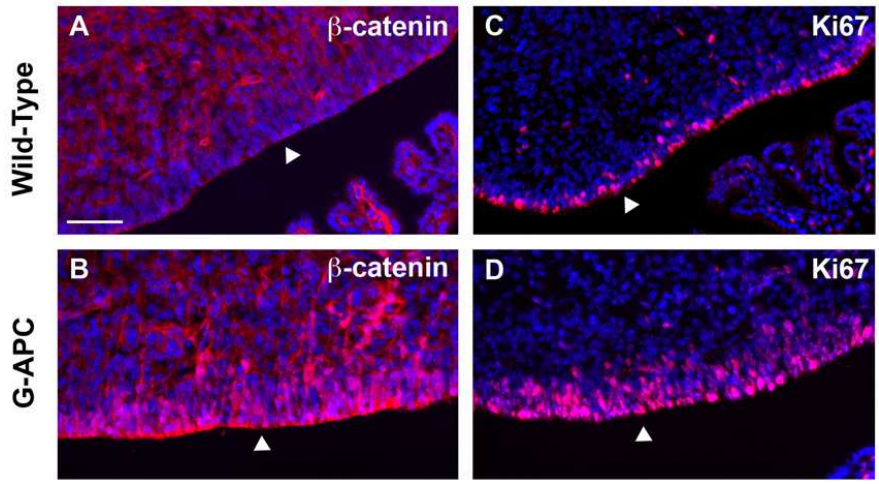

Figure 9: Inactivation of Apc in the cerebellar ventricular zone leads to WNT pathway activation and proliferation.

(A-D**) Cerebellar sections from E14.5 WT (A,C) and G-APC (B,D) mice were stained with DAPI (blue) and anti-β-catenin (red, A,B) or anti-Ki67 (red, C,D) antibodies to detect WNT pathway activation and proliferation, respectively. Note the accumulation of nuclear β-catenin (colocalization with DAPI in B) and increased proliferation (Ki67^{+} cells in D) in the VZ of G-APC mice. Arrowheads indicate VZ. Scale bar: 50 µm. SNB.**

β-catenin in the VZ (Fig. 9A,B). Similar to G-Cat mice, G-APC mice showed increased proliferation in the VZ at E14.5 (Fig. 9C,D). G-APC mice also died shortly after birth

andhad much smaller cerebella than WT littermates at P0 (Fig. 10). Moreover, whereas WT cerebella contain proliferating (Ki67+) Pax6+ GNPs on their surface (Fig. 10A,C), mutant cerebella had few such cells (Fig. 10B,D). As with G-Cat mice, staining of G-APC cerebella with anti-calbindin antibodies revealed disorganization of Purkinje neurons (Fig. 10E,F). Staining for Pax2 (Fig. 10G,H) and S100β (Fig. 10I,J) also showed marked reductions in the numbers of interneurons and glia in mutant cerebella. These results suggest that WNT pathway activation – either through stabilization of β -catenin or loss of Apc – causes increased proliferation of NSCs and defective differentiation of these cells into neuronal and glial progenitors.

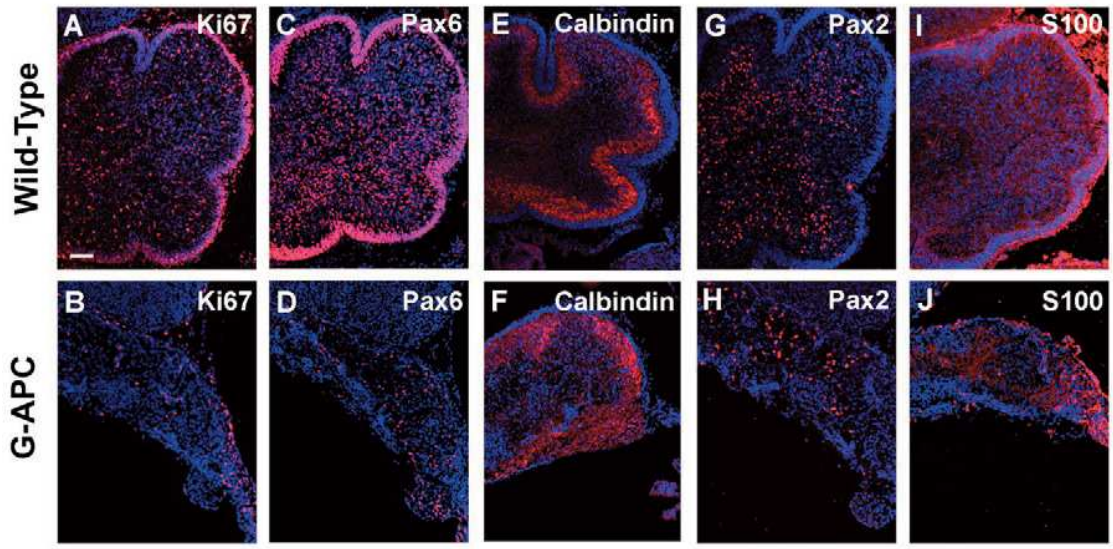

Figure 10: Deletion of Apc in stem cells also impairs differentiation.

(A-J) Sections from P0 WT (A,C,E,G,I) and *hGFAP-Cre; Apc*$^{lox/lox}$ (G-APC; B,D,F,H,J) cerebella were stained with antibodies specific for Ki67 (A,B) to label proliferating cells, Pax6 (C,D) to label GNPs, calbindin (E,F) to mark Purkinje neurons, Pax2 (G,H) to detect stellate and basket interneuron precursors, and S100β (I,J) to detect glia cells. Sections were counterstained with DAPI. Note the marked reduction in progenitors and differentiated cells in G-APC mice. Scale bar: 50μm. SNB.

2.2.5 Overexpression of β-catenin impairs NSC self-renewal and differentiation *in vitro*

The above studies suggest that WNT pathway activation causes cerebellar NSCs to proliferate but does not result in increased numbers of differentiated cells. To understand the basis for this defect, we examined the effects of β-catenin on self-renewal. Cells were isolated from WT and G-Cat mice at P0 and cultured in serum-free medium containing bFGF (FGF2) and EGF. As we have shown previously[31], Prom1+ Lin– cells from WT mice form neurospheres under these conditions (Fig. 11A,C). By contrast, NSCs from mutant cerebellum formed neurospheres at a significantly lower frequency (P<0.01; Fig. 11B,C). Moreover, mutant neurospheres were smaller and could not give rise to secondary neurospheres, indicating a defect in self renewal. Similar results were observed when NSCs were isolated from the cerebellum at E14.5 (data not shown). Thus, constitutive activation of β-catenin in GFAP+ cells causes transient proliferation of NSCs but impairs their ability to undergo self-renewal. This defect in self-renewal could explain the reduction in cerebellar mass in these mice.

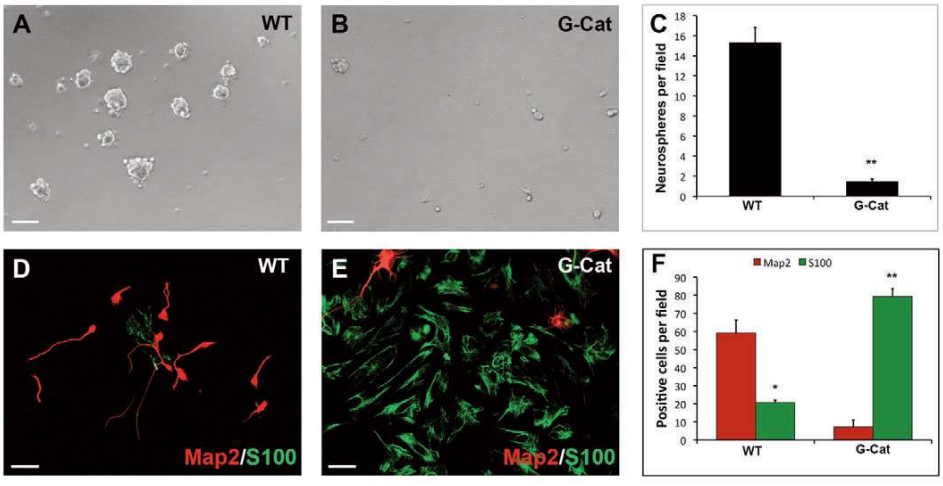

Figure 11: β-catenin-expressing stem cells exhibit aberrant self-renewal and differentiation *in vitro*.

(A,B) Prom1+ Lin– cells were sorted from P0 WT (A) or G-Cat (B) mouse cerebellum and cultured at clonal density in serum-free medium containing 25 ng/ml EGF and bFGF for 7 days before being photographed under bright-field. Passageable neurospheres were consistently obtained from WT but not from mutant cerebellum. (C) Average neurosphere numbers (±s.e.m.) per field from eight fields in P0 WT or G Cat cerebellum. (D,E) Prom1+ Lin– cells were sorted from P0 WT (D) or G-Cat (E) cerebellum and cultured for 3 days in medium containing 1% FBS. Cells were stained with antibodies specific for the neuronal marker Map2 (red) and the glial cell marker S100β (green). (F) Quantitation (mean ± s.e.m.) of Map2/S100β staining from five fields. *, *P*<0.01; **, *P*<0.001. Whereas WT stem cells differentiated predominantly into neurons, G-Cat showed a marked skewing toward glial lineages. Scale bars: 25μm in A,B; 50μm in D,E. YX and SNB.

We next asked whether WNT signaling affects the differentiation of cerebellar NSCs *in vitro*. Although NSC differentiation is often assayed by generating neurospheres and then withdrawing growth factors, the impaired neurosphere-forming capacity of mutant NSCs precluded such analysis. Instead, we isolated Prom1+ Lin– cells from P0 WT and mutant mice and cultured them in the presence of 1% FBS for 3 days. Cells were stained with antibodies specific for Map2 (Mtap2 – Mouse Genome Informatics; to detect neuronal differentiation) and S100β (to detect glial differentiation). Whereas NSCs from WT animals predominantly generated neurons (60% Map2+, 22% S100 β +), NSCs from mutant mice almost exclusively generated glia (7% Map2+, 80% S100 β +) (Fig. 11D-F). These data, which are consistent with the marked depletion of neurons in G-Cat mice *in vivo*, suggest that activation of β-catenin in GFAP+ cells markedly alters the differentiation potential of postnatal cerebellar NSCs.

2.2.6 Loss of β-catenin causes self-renewal defects and increased neuronal differentiation *in vitro*

Given the effects of β-catenin overexpression on stem cell self-renewal and differentiation, we examined the effects of loss of β- catenin on these processes. We used *Catnb*$^{lox(ex2\text{-}6)}$ mice, in which exons 2-6 of β-catenin are flanked by loxP sites.

Introduction of Cre into these cells results in excision of exons 2-6 and loss of β-catenin expression. Prom1+ Lin– cells were isolated from *Catnb*$^{lox(ex2-6)}$ mice at P0, infected with control (GFP) viruses (Fig. 12A) or Cre-IRES-GFP viruses (Fig. 12B) and cultured in stem cell media in the presence of bFGF and EGF. After 10 days, total and GFP-expressing neurospheres were counted. The percentage of GFP+ neurospheres was significantly lower in Cre-infected cultures than in control cultures (Fig. 12A,C). Additionally, the neurospheres from Cre-infected cells were smaller and more irregularly shaped than those from control cells (Fig. 12B,C). The fact that both gain and loss of β-catenin result in decreased neurosphere-forming capacity suggests that the level of β-catenin is crucial for maintenance of self-renewal capacity.

We then asked whether loss of β-catenin affects the differentiation of stem cells. Prom1+ Lin– cells were isolated from *Catnb*$^{lox(ex2-6)}$ mice, infected with viruses, and maintained in 3% FBS-containing media for 3 days to allow for differentiation. Cells were then stained for Map2 (Fig. 12D,E) and S100 β (Fig. 12F,G) to mark differentiated

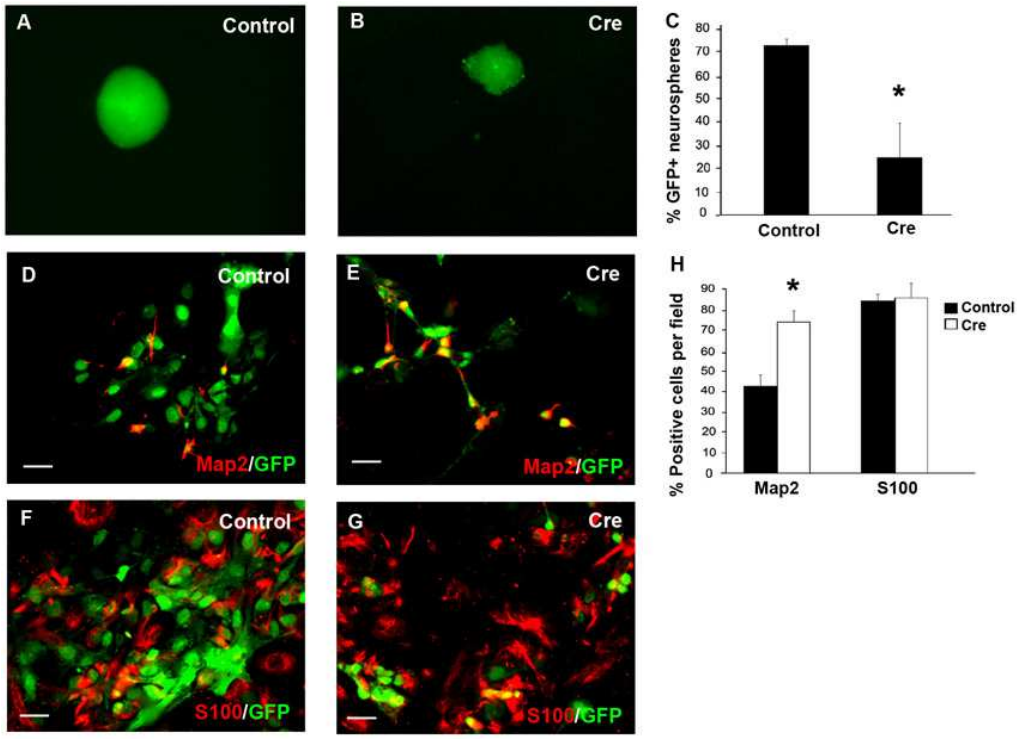

Figure 12: **Loss of β-catenin impairs self-renewal and increases neuronal differentiation.**

(A-C**) Prom1^{+} Lin^{-} cells were isolated from P0 *Catnb^{lox}(^{ex3})$^{/+}$* mice and infected with control (GFP) (A) or Cre-IRES-GFP (B) viruses overnight. Cells were collected and replated at low density (2000 cells/ml) and cultured for 7 days under neurosphere-promoting conditions. (A,B) Representative images of GFP-expressing neurospheres. (C) Total neurospheres and GFP^{+} neurospheres were counted for each condition. The percentage of GFP^{+} neurospheres was calculated from five replicates and significance was determined using *t*-test. (**D-G**) Prom1^{+} Lin^{-} cells were isolated from Catnb^{lox}(^{ex3})$^{/+}$ mice and infected with control (D,F) or Cre-IRES-GFP (E,G) viruses. Cells were fixed after 3 days in culture and stained with antibodies against Map2 (D,E) to mark neuronal cells or S100 (F,G) to label glial cells. (**H**) Percentage of GFP/Map2 and GFP/S100 were calculated from three replicates. *, *P*<0.01. Scale bar: 50 µm. SNB.**

neuronal and glial cells. Interestingly, we found an increase of Map2+ cells among Cre-infected cells compared with control cells (Fig. 12D,E,H). Loss of β-catenin had no effect on the percentage of S100 β + cells as compared with control cells (Fig. 12F-H). These

data suggest that loss of β-catenin skews stem cell differentiation toward neuronal lineages.

2.2.7 Activation of β-catenin causes increased expression of BMPs and p21

Our studies suggested that WNT signaling promotes the proliferation of cerebellar NSCs but interferes with their capacity for self-renewal and differentiation. To gain insight into the molecular basis for these findings, we isolated RNA from WT and mutant Prom1+ Lin– cells and analyzed expression of WNT target genes and regulators of proliferation and self-renewal. RT-QPCR analysis revealed no consistent differences in expression of the polycomb transcription factor *Bmi1* or the Notch target genes *Hes1* and *Hes5* (Fig. 13A), each of which has been implicated in NSC self-renewal[120-122]. By contrast, expression of *c-Myc*, a common target of the WNT pathway[123,124], was elevated in NSCs isolated from G-Cat mice (Fig. 13A).

Although increased expression of *c-Myc* provided a potential explanation for the increase in proliferation induced by β-catenin, the basis for the defects in self-renewal and differentiation in G-Cat mice remained unclear. Previous reports have suggested that in neuroepithelial progenitors from the dorsal CNS, WNT signaling not only induces proliferation but also increases the expression of bone morphogenetic proteins (BMPs)[106]. BMPs, in turn, inhibit WNT induced proliferation and self-renewal[106] and promote glial differentiation[125,126]. This led us to examine whether the self-renewal and differentiation defects in G-Cat mice are mediated by BMPs. As shown in Fig. 11A, mutant cells exhibited a significant increase in the expression of *Bmp2* and *Bmp7*. In addition, expression of the cyclin-dependent kinase inhibitor *p21* (*Cdkn1a* – Mouse Genome Informatics), which is a common target of BMPs, was also markedly elevated in

NSCs from G-Cat mice. These findings suggest that overexpression of β-catenin in NSCs results in increased BMP/p21 signaling.

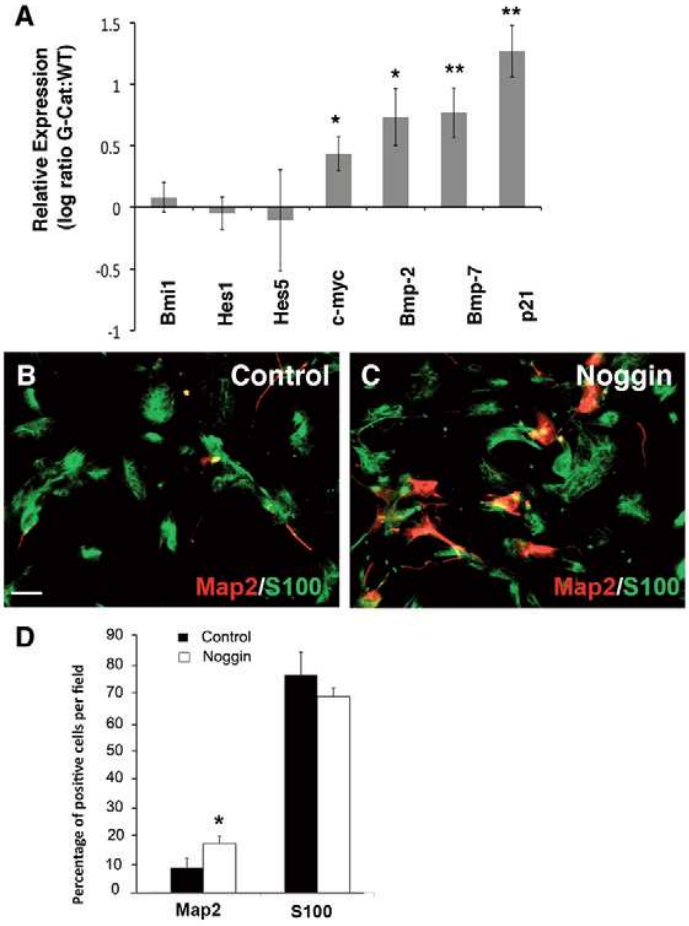

Figure 13: Stem cells from G-Cat mice express elevated levels of BMPs and p21.

(A) Quantitative RT-PCR was performed on Prom1+ Lin– cells isolated from P0 WT and G-Cat mice using primers for the indicated genes. Mice from four litters were analyzed, and after normalization to actin the expression of each gene in G-Cat cells was compared with that in WT littermates to calculate a ratio of gene expression for each litter. Data are plotted as the mean (±s.e.m.) log ratio (G-Cat:WT). *, *P*<0.02; **, *P*<0.05. (B-D) Noggin promotes neuronal differentiation. Prom1+ Lin– cells were isolated from P0 G-Cat mice, treated with noggin protein (200 ng/ml) and cultured for 3 days in serum-containing media to promote differentiation. Cells were stained for Map2 (red) and S100β (green). Quantitation

(mean ± s.e.m.) of Map2/S100β staining is from six fields. *, *P*<0.01. Scale bar: 50μm. SNB.

To determine whether the BMP pathway regulates self-renewal of cerebellar stem cells, we performed both gain- and loss-of function experiments. Prom1+ Lin– cells from WT mice were cultured at low density in the presence of growth factors along with recombinant Bmp2 or Bmp7 (200 ng/ml). After 7 days, stem cells treated with either Bmp2 or Bmp7 showed a marked decrease in neurosphere formation compared with control cells (Fig. 14A). Conversely, Prom1+ Lin– stem cells treated with the BMP inhibitor noggin produced more neurospheres than control cells (Fig. 14B). Cells infected with viruses encoding short hairpin RNA specific for *p21* (p21shRNA) also showed increased neurosphere formation (Fig. 14B). These studies suggested that BMP signaling interferes with the self-renewal of cerebellar stem cells.

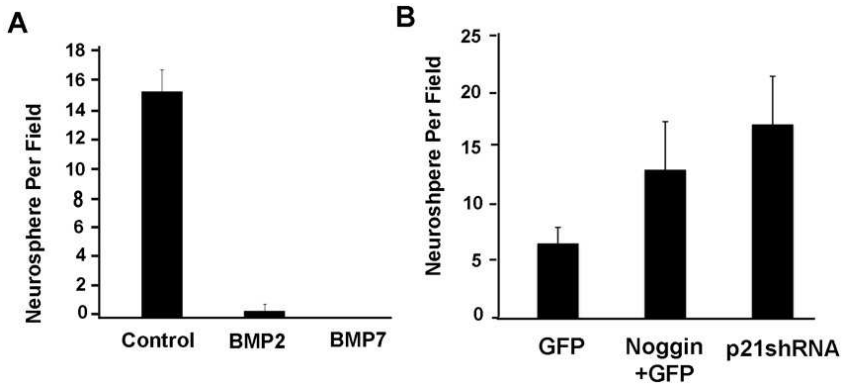

Figure 14: BMP signaling impairs stem cell self-renewal *in vitro*.

(A**) WT Prom1^{+} Lin^{-} cells were isolated at P0 and plated at low density for 7 days in serum-free media containing bFGF and EGF, without (control) or with BMP2 (200 ng/ml) or BMP7 (200 ng/ml). (**B**) WT Prom1^{+} Lin^{-} cells from P0 WT mice were infected with GFP viruses alone (GFP), infected with GFP viruses and treated with noggin (200 ng/ml), or infected with p21shRNA viruses (without noggin) and cultured in serum-free medium containing bFGF and EGF. After 7 days, noggin-treated cells or p21-infected cells generated 2- to 2.5-fold more neurospheres than GFP-infected cells. SNB.**

To determine whether increased BMP signaling could explain the impaired self-renewal and differentiation of stem cells from G-Cat mice, we isolated Prom1+ Lin– cells from P0 G-Cat mice and treated them with noggin or p21shRNA. Neither noggin treatment nor p21 knockdown was able to rescue the neurosphere defect in these cells (data not shown). However, noggin significantly increased neuronal differentiation and slightly decreased glial differentiation of G-Cat cells (Fig. 13B-D). Knockdown of *p21* promoted the neuronal differentiation of WT stem cells (Fig. 15AE), although it was not able to rescue the differentiation defects in G-Cat stem cells (Fig. 15F-J). Together, these data suggest that the BMP pathway regulates the self renewal and differentiation of WT stem cells, but is not sufficient to account for the defects in self-renewal and differentiation seen in β –catenin overexpressing mice.

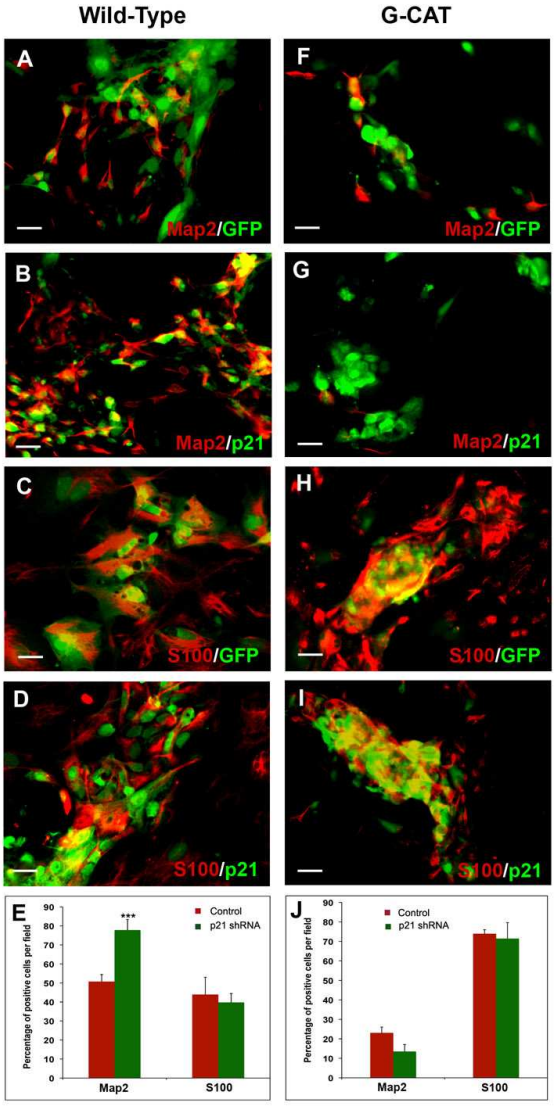

Figure 15: Knockdown of p21 promotes neuronal differentiation *in vitro*.

(A-D,F-I**) Prom1^{+} Lin^{-} cells from P0 WT or G-Cat mice were infected with GFP (A-D) or Cre-IRES-GFP (F-I) viruses and cultured for 3 days in serum-containing medium to promote differentiation. Cells were stained for Map2 (red) or S100 (green). (**E**) Quantification of Map2/GFP^{+} or S100/GFP^{+} cells from WT stem cells. (**J**) Quantification of Map2/GFP^{+} or S100/GFP^{+} cells from G-Cat stem cells. Scale bar: 50 µm. YX and SNB.**

2.3 Discussion

WNT signaling plays a crucial role in the development of many parts of the CNS and, in particular, has been implicated in the establishment of the midbrain hindbrain boundary that gives rise to the cerebellum. The fact that a subset of human medulloblastomas contains activating mutations in β -catenin or loss-of function mutations in APC suggests that WNT signaling might also be mitogenic for a population of cells in the developing cerebellum. To identify such a population, we screened cells from various stages of cerebellar development for WNT responsiveness. Our studies suggest that NSCs from the embryonic and neonatal cerebellum are capable of proliferating in response to WNT pathway activation. However, the increased proliferation of these cells *in vivo* is accompanied by marked defects in self-renewal and differentiation. Based on these studies, we conclude that WNT signaling induces proliferation but impairs differentiation of cerebellar stem cells.

Although WNT signaling is known to be mitogenic for NSCs in other parts of the CNS[102-104], it has not been shown to promote proliferation in the cerebellum. We observed WNT pathway induced proliferation both *in vitro*, upon treatment of NSCs with WNT protein or infection with β -catenin-encoding retroviruses, and *in vivo*, following activation of β-catenin or deletion of *Apc* using the hGFAP-Cre transgene. It is interesting that in previous studies examining the effects of WNT pathway activation in the CNS, there was no mention of mitogenic effects in the cerebellum[102-104]; it is unclear whether these studies did not examine this region, or whether the promoters used [nestin, *Brn4* (*Pou3f4*)] did not target the same population of NSCs at the same stage of development. However, recent studies have demonstrated that deletion of β-catenin results in loss of the cerebellar vermis[74], and these investigators speculated that this

might result from inadequate proliferation. Our studies support this notion and suggest that, at least early in development, the mitogenic effects of WNT signaling might contribute to expansion of the cerebellar primordium.

Although activation of WNT signaling promotes proliferation of NSCs, this proliferation appears to be transient: G-Cat mice exhibit increased numbers of dividing Sox1+ cells in the VZ at embryonic stages, but these cells do not expand indefinitely *in vivo* and cannot form neurospheres *in vitro*. One explanation for the failure of mutant NSCs to form neurospheres is that activation of the WNT pathway allows cells to proliferate at high density but does not favor growth under neurosphere-forming conditions (at low density in EGF and bFGF). Alternatively, it is possible that aberrant expression of β-catenin affects the cell surface expression of cadherins and thereby interferes with cell-cell interactions that are necessary for cells to grow as neurospheres[127] . Finally, WNT pathway activation, while promoting proliferation, might change the mode by which NSCs divide; for example, by shifting the balance from symmetric renewal (whereby a stem cell gives rise to two stem cells) or asymmetric division (whereby stem cells give rise to one stem cell and one differentiated cell) to symmetric differentiation (wherein both progeny undergo differentiation). This might explain both the inability to form neurospheres *in vitro* and the lack of prolonged expansion *in vivo*.

In addition to its effects on proliferation and self-renewal, WNT pathway activation also severely disrupts NSC differentiation. *In vitro*, this is manifest as a marked skewing toward glial cells at the expense of neuronal lineages. *In vivo* the effects are even more striking, with a reduction in most classes of neurons and a decrease in the overall size of the cerebellum. In principle, these defects could reflect

either a failure of NSCs to commit to neuronal lineages or the impaired growth and survival of neuronal progenitors or neurons once they are formed. The fact that few Purkinje and granule neurons can be detected in the cerebellum of G-Cat mice, and that purified NSCs from these animals differentiate predominantly into S100 β + glia, support the former possibility. However, our observations that M-Cat mice have a normal EGL but produce fewer GNPs suggest that impaired proliferation might also contribute to the reduction in the number of neurons, at least in the granule lineage. Consistent with this, a recent study has demonstrated that loss of Apc in GNPs results in inhibition of cell proliferation[128]. Although WNT signaling promotes neurogenesis in many parts of the nervous system[103,108,129], its ability to interfere with neuronal differentiation in the cerebellum is not without precedent. For example, β -catenin has been reported to inhibit neuronal differentiation in embryonic stem cells and in progenitors within the spinal cord[106,130]. Likewise, in the hematopoietic system, activation of β -catenin increases proliferation of stem cells[131,132] but can also cause a profound block in differentiation along myeloid, lymphoid and erythroid lineages[133,134]. Finally, stabilization of β catenin in the airway epithelium blocks Clara cell differentiation[135]. Thus, impaired differentiation is not an uncommon effect of WNT pathway activation.

To understand the mechanisms by which β-catenin alters NSC renewal and differentiation, we examined expression of genes that have been identified as targets of the WNT pathway in other cells. Our results showed that *c-Myc*, a common target of WNT signaling[123], was elevated in NSCs from G-Cat mice compared with control NSCs. Increased expression of *c-Myc* could account for the NSC proliferation we observed in embryonic and postnatal G-Cat mice, but is difficult to reconcile with the defects in self-renewal and differentiation observed in these animals. In this regard, our finding that

NSCs from G-Cat mice express increased levels of *Bmp2* and *Bmp7* might be important. BMPs have been reported to be induced by β-catenin and to counteract the mitogenic effects of canonical WNT signaling in the dorsal neural tube[106]. In addition, BMPs frequently induce expression of the cyclin-dependent kinase inhibitor p21[136-138] which has also been shown to inhibit self renewal of NSCs[139,140]. The fact that we observe significant upregulation of both *BMP*s and *p21* raises the possibility that this pathway contributes to the self-renewal defects in G-Cat mice. However, our inability to rescue these defects with noggin or p21shRNA implies that other pathways might be involved as well. Notably, in addition to their inhibitory effects on proliferation, BMPs are also potent inducers of glial differentiation[125,141]. Thus, increased expression of *BMPs* might also explain the skewing of NSC differentiation toward the glial lineage that we observed *in vitro* and *in vivo*. Our observation that noggin can restore neuronal differentiation of G-Cat stem cells supports a role for BMP signaling in this process.

Although G-Cat mice do not live long enough to develop brain tumors, our studies have implications for understanding WNTassociated medulloblastoma. First, our observation that β-catenin does not promote the proliferation of GNPs *in vitro* or *in vivo* suggests that unlike Shh-associated tumors[115] WNT-associated tumors are unlikely to arise from GNPs. By contrast, the fact that WNT is mitogenic for NSCs in the cerebellar VZ and postnatal white matter raises the possibility that these represent cells of origin for the disease. The idea that SHH- and WNT-driven tumors come from different cells of origin is supported by genomic analysis[14,18,142,143], which indicates that these tumors have very distinct gene expression profiles. Although recent studies have shown that WNT-associated tumors can arise from progenitors in the dorsal brainstem[24], our results

raise the possibility that some of these tumors could also originate from embryonic or postnatal NSCs in the cerebellar VZ.

Whatever the cell of origin for WNT pathway tumors, it seems likely that activation of WNT signaling alone is insufficient to drive tumorigenesis. This is supported by our own studies, in which activation of β-catenin or deletion of *Apc* drives proliferation but not long-term self-renewal of NSCs. Similarly, activation of β-catenin in BLBP-expressing cells does not cause tumors on its own, but can synergize with loss of p53 (Trp53) to promote medulloblastoma formation[24]. Interestingly, analysis of human WNT pathway tumors indicates that they frequently lose one copy of chromosome 6[13,14], suggesting that loss of a gene on this chromosome might be a key event in tumorigenesis. Identification of WNT responsive cells in the cerebellum will facilitate analysis of specific genes that may cooperate with the WNT pathway to promote uncontrolled growth or tumor formation.

3. Survivin as a therapeutic target in Sonic hedgehog-driven medulloblastoma

3.1 Introduction

As was introduced in previous chapters, medulloblastoma (MB) is the most common malignant brain tumor in children[1]. Intensive therapy –including surgery, cranio-spinal radiation and high dose chemotherapy – has improved 5-year survival rates[2], but almost a third of MB patients still die from their disease, and survivors suffer severe long term side effects that affect their quality of life[23]. Thus, safer and more effective therapies are needed for this disease.

In recent years, targeted therapies have begun to be evaluated in patients with MB. For example, with the recognition that a subset of MBs results from mutations in the Sonic Hedgehog (SHH) pathway[143], antagonists of Smoothened (SMO), an activator of the pathway, have advanced into clinical trials for the disease. Although initial reports suggested that these agents can inhibit tumor growth[20], as with many targeted therapies, resistance develops quickly[144]. Moreover, patients who have SHH pathway mutations downstream of SMO do not respond to these agents at all[145]. Thus, even for SHH-driven MBs, additional approaches are necessary.

Finding novel therapeutic targets for MB depends on identification of genes that are critical for tumor growth and survival. One gene that is highly expressed in human MB and could represent a potential new therapeutic target is Survivin (also known as baculoviral inhibitor of apoptosis repeat-containing 5, or *BIRC5)*.

Survivin was first identified in human B cell lymphomas[146] and is a member of the IAP family that regulates both cell cycle progression and cell survival[147,148]. It contains one baculovirus IAP repeat (BIR), which is a hallmark of all IAPs and is required for their

ability to inhibit apoptosis. Survivin lacks any of the other functional domains commonly found in IAPs, such as a caspase recruitment domain[149,150]. Survivin is ubiquitously expressed during embryonic and fetal development, but is undetectable in most differentiated adult tissues[151]. Germline knockout in the mouse leads to lethality at E3.5, supporting an essential role in development[152]. Tissue specific knockouts have revealed important functions for Survivin in development of pancreatic beta cells[153,154] and endothelial cells[155], and deletion in neural progenitors results in decreased brain size and perinatal lethality, suggesting critical roles in neural development as well[156]. Recent studies have suggested a role for survivin in a restricted set of proliferative adult tissues as well[157], namely CD34+ hematopoietic stem cells[158], T lymphocytes[159,160], neutrophils[161], and vascular endothelial cells[162,163].

In context of the cell cycle, Survivin functions primarily as a critical component of the chromosomal passenger complex (CPC), which includes Survivin, Aurora B, INCENP (inner centromere protein) and Borealin[164]. The CPC binds to centromeres and corrects the alignment of chromosomes at metaphase, and then migrates to the spindle midzone in late mitosis to enable completion of cell cleavage.[164-166] If Survivin (or any of the other CPC proteins) is suppressed, the complex does not associate with the centromere, and the metaphase spindle assembly checkpoint is abrogated[164,167]. .Survivin in is critical for mediating localization of this complex throughout mitosis and subsequent activation of Aurora B by its ability to recognize and bind phosphorylated histone H3 (Thr3), which recruits the CPC to chromosomes [168-171]. In addition, absence of CPC proteins interferes with cell cleavage[172]. Thus, Survivin function is critical for mitosis. It has also been suggested that Survivin regulates microtubule dynamics, possibility independent of its participation in the CPC[173,174]. Finally, Survivin contributes

to regulation of the G1-S transition in T lymphocytes[159], hematopoietic progenitors[175,176], and hepatoma cells[177], although the mechanism is not as well understood.

Consistent with structural similarity to other IAPs, survivin is also involved in cell survival and apoptosis inhibition. Overexpression of survivin is associated with inhibition of cell death via both intrinsic and extrinsic apoptosis pathways *in vitro* and cell death inhibition *in vivo*[178-183]. Similarly, loss of survivin expression/function sensitizes cells to various apoptotic stimuli, including FAS ligand, overexpression of BAX, p53, caspases-3, -7, -8, and cytotoxic drugs[184-187]. Although initial data suggested that this involved direct inhibition of caspase activity[185,188] (the effector enzymes of apoptosis) current evidence indicates that, as with most IAPs, Survivin regulates their activity indirectly[189-192]. The cytoprotective effect of survivin instead arises from interactions with cofactors or effectors. Survivin can form a complex with HBXIP (Hepatitis B X-interacting protein), which then binds to pro-caspase 9 and prevents its recruitment to the apoptosome[193]. It can also enhance the activity of other IAPs, either through direct binding to XIAP (X-linked inhibitor of apoptosis)[194] or by interacting with SMAC-Diablo (second mitochondria-derived activator of caspases/direct IAP binding protein with low pI), which displaces SMAC-bound IAPs, allowing them to inhibit apoptosis[195,196]. A subfraction of survivin is also imported to the mitochondria, where it can bind proapoptotic factors and inhibit mitochondrial apoptosis[185].

In addition to its roles in normal development, survivin seems to be an important player in tumorigenesis. Survivin is expressed in many human cancers (both adult and pediatric) with minimal expression in normal adult differentiated tissues[147], making a highly cancer-specific marker. Importantly, its expression is correlated with poor clinical outcome in numerous malignancies, including non-small cell lung cancer (NSCLC)[197],

breast cancer[198], leukemia[199,200], neuroblastoma[201] and glioma[202]. Recently, it has also been identified as a biomarker for bladder[203] and breast cancer[204,205] and to be a critical mediator of early stages of tumor development and progression in liver and prostate cancer[206,207]. Additionally, Survivin interacts with multiple signaling networks critical for tumorigenesis, making it a nodal protein and ideal target for therapies. For example, Survivin expression is regulated by numerous oncogenes and tumor suppressors commonly mutated in cancer such as p53[181,208], Ras[209], and Myc[210] and binding sites for transcription factors such as Stat3, E2F proteins, and NFkB have been mapped to the Survivin promoter[201]. Survivin has also been implicated in promoting angiogenesis in tumors[211,212] and inhibition of survivin in ECs by antisense oligonucleotide[163] or introduction of a mutant form of survivin[67] causes vascular regression during tumor angiogenesis. Because of its high expression in tumors and minimal expression in most adult tissues[151], a number of approaches have been developed to inhibit its expression and function. These include small molecule antagonists, dominant negative mutants, antisense oligonucleotides, and immunotherapy[213-217]. Numerous studies across cancer types have demonstrated the efficacy of inhibiting Survivin on impeding tumor growth and survival both *in vitro* and *in vivo*[147]. Some of these targeting approaches have now advanced to phase 1 and 2 clinical trials for a range of tumor types, including prostate cancer[218,219], lymphoma[219], leukemia[220], melanoma[221-223], breast cancer[223,224], and NSCLC[223-225].

Survivin has not yet been studied extensively in the context of MB. Although some studies have suggested that elevated expression is linked to poor prognosis[226-228], little is known about its role in MB growth and survival. Using an animal model of SHH-driven MB, we now show that Survivin is highly expressed in tumors and absent from

normal adult cerebellum. Moreover, through both genetic deletion and pharmacological inhibition we demonstrate that Survivin is critical for proliferation and survival of mouse and human SHH driven MB cells. Finally, Survivin antagonists impair growth of MB *in vivo*, highlighting the potential of Survivin as a therapeutic target in patients with MB.

3.2 Results

3.2.1 Survivin is highly expressed in medulloblastomas from *Ptch* mutant mice

To determine whether Survivin could represent a target in SHH driven MB, we isolated RNA from *Ptch* mutant tumors and examined *survivin* expression using real time PCR. High levels of *survivin* were detected in all tumors and in granule neuron precursors (GNPs), the progenitors from which these tumors are thought to arise[115] (Figure 16A). Importantly, expression could not be detected in normal adult cerebellum. Similar results were seen when Survivin protein was examined by immunoblotting (Figure 16B). Staining of tissue sections revealed Survivin expression in the nuclei of tumor cells (abrogated by blocking peptide (Figure 16D)), and minimal staining in normal adult cerebellum (Figure 16C-F). These data indicate that Survivin is highly expressed in *Ptch* mutant tumors, raising the possibility that it might play an important role in tumor growth or maintenance.

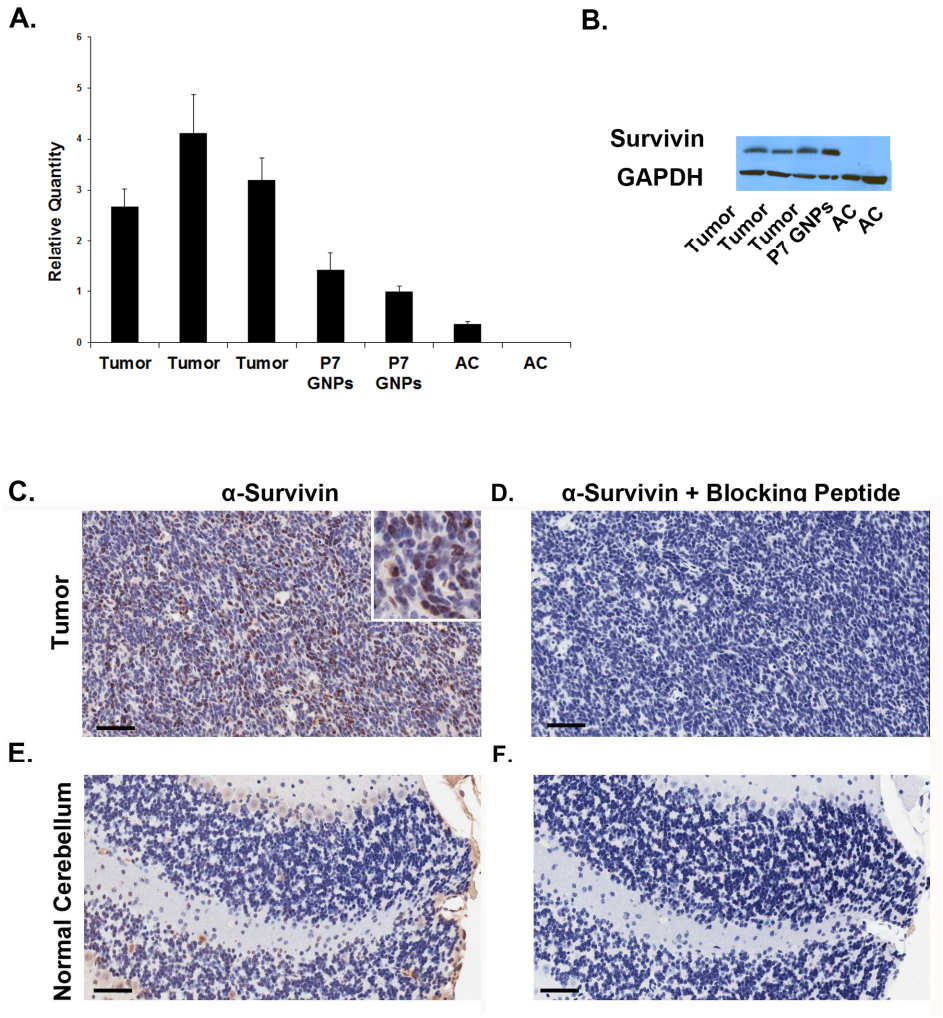

Figure 16: Survivin is expressed in Ptch mutant tumors.

(A) RNA and protein from *Ptch* mutant MB cells, P7 GNPs and adult cerebellum were analyzed for Survivin expression using real time PCR (A) and by western blotting (B). Survivin is highly expressed in tumors and GNPs, but not in adult cerebellum. Error bars in (A) represent 95% confidence interval calculated using sum of the squares method ($p<0.02$ by ANOVA and post hoc student's t-test). (C-F) Tissue sections from *Ptch* mutant tumor and normal adult cerebellum were stained with anti-Survivin antibodies alone (C,E) or with anti-Survivin antibodies that were pre-incubated for 30 min with Survivin blocking peptide (D,F). Survivin is highly expressed in tumor cells with minimal expression in adult cerebellum. Inset in (C) is 4x magnification of positive staining. Scale bars represent 50 μM. Data are representative of 3 experiments.

3.2.2 Survivin is critical for MB cell proliferation and cell cycle progression

To investigate the importance of Survivin for growth of MB cells, we first utilized a genetic approach. *Survivin*$^{fl/fl}$ mice[160], in which the *survivin* gene is flanked by loxP sites, were crossed with *Ptch*$^{+/-}$ mice, to generate tumors in which *survivin* can be deleted by Cre recombinase. We confirmed efficient deletion of *survivin* by isolating tumor cells from *Survivin*$^{fl/fl}$*;Ptch*$^{+/-}$ (SP) mice and infecting them with Cre retroviruses. After 48hrs, *survivin* expression was significantly reduced (by 82%) in Cre-infected cells compared to control (GFP-infected) cells (Figure 17A). We then looked at the effect of *survivin* loss on proliferation. After Cre-mediated deletion of *survivin* from SP tumor cells, thymidine incorporation was decreased by almost 90% (Figure 17B). Importantly, when tumor cells from *Survivin*^{WT} mice were infected with Cre viruses, there was no appreciable difference in proliferation compared to control cells (Figure 17C), indicating that the decreased thymidine incorporation observed in SP tumor cells was not due to non-specific toxicity of the Cre virus. Similarly, loss of survivin in normal GNPs caused a decrease in proliferation. (Figure 18A-C). To address whether loss of *survivin* affects cell cycle progression, we isolated cells from SP tumors, infected them with Cre or GFP viruses, and performed cell cycle analysis (Figure 17D,E). *survivin* deletion led to a marked accumulation of cells in the G2/M phases of the cell cycle (39% of Cre-infected cells vs. 9.5% of control cells in G2/M). Together, these data demonstrate that Survivin is necessary for proliferation and cell cycle progression of MB cells.

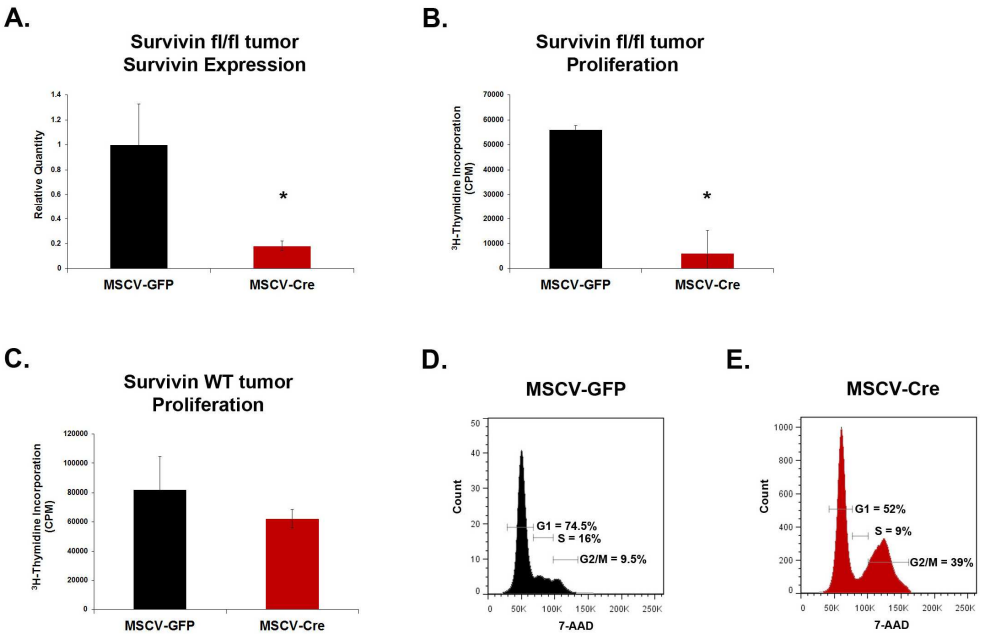

Figure 17: Loss of Survivin causes decreased proliferation and cell cycle arrest.

(A-B) Cells were isolated from *Survivin*$^{fl/fl}$;*Ptch*$^{+/-}$ tumors and infected with Cre- or GFP retroviruses for 48 hr. (A) GFP+ cells were isolated by flow cytometry and *survivin* mRNA expression analyzed by qRT-PCR (n=2). Cre causes loss of *survivin* expression ($p<0.02$). (B) Cells were pulsed with ^{3}H-thymidine for 12 hr, harvested, and analyzed for incorporation. Loss of *survivin* leads to decreased tumor cell proliferation ($p<0.001$). Data are representative of 5 experiments. (C) Cells were isolated from *Ptch*$^{+/-}$ tumor (wild type for survivin), infected with Cre- or GFP viruses for 48 hr, and collected after 12 hr pulse with ^{3}H-thymidine to measure incorporation. Infection with Cre virus alone does not significantly impair proliferation ($p>0.1$). Data in (A-C) represent mean +/- standard deviation (SD) and are representative of 4 experiments. (D, E) Cells from *Survivin*$^{fl/fl}$;*Ptch*$^{+/-}$ tumors were infected with virus as described above (D. GFP, E. Cre virus) and stained with 7-AAD for cell cycle analysis by flow cytometry. *survivin* deletion causes accumulation of cells in G2/M. Data are representative of 4 experiments and cell cycle percentages based on live cell gates (excluded subG1). p values calculated using student's t-test.

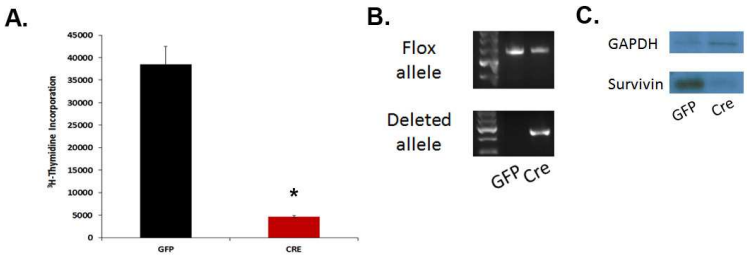

Figure 18: Survivin deletion inhibits proliferation of GNPs.

(A-C) GNPs were isolated from P7 wildtype cerebella and infected with Cre or GFP expressing viruses for 48hrs. (A) Cells were pulsed with ^{3}H-thymidine for 12 hr, harvested, and analyzed for incorporation. Loss of *survivin* leads to decreased GNP proliferation ($p<0.001$). (B-C) Infected GNPs were analyzed for *survivin* deletion by PCR (B) and western blotting (C). *survivin* is deleted in GNPs following Cre infection. (C) Error bars are +/-SD and data are representative of 2 experiments.

3.2.3 Survivin antagonists inhibit MB cell proliferation and promote apoptosis

Given the importance of Survivin for MB proliferation, we hypothesized that pharmacological agents that inhibit Survivin expression or function might interfere with tumor growth. To test this, we obtained several small molecule Survivin antagonists: YM155 is an inhibitor of *survivin* transcription[229], whereas S12 and LLP3 bind directly to Survivin protein and interfere with its function[230,231]. To test the ability of YM155 to inhibit *survivin* expression in *Ptch* mutant MB cells, we treated cells with the drug for 48hrs, isolated RNA and performed qRT-PCR for *survivin*. YM155 markedly decreased *survivin* expression even at a concentration of 10 nM (Figure 19A). Similarly, loss of Survivin was detected at the protein level using western blotting (Figure 19B). These data suggest that YM155 effectively inhibits *survivin* expression in *Ptch* mutant MB cells.

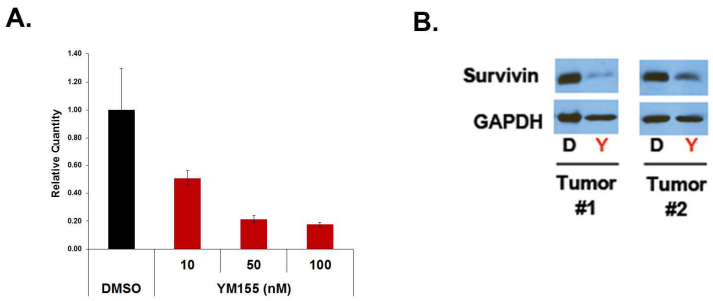

Figure 19: YM155 inhibits survivin expression in MB cells.

***Ptch* mutant tumor cells were treated with DMSO (D) or YM155 (Y) for 48 hours and analyzed for expression of *survivin* by real time PCR (A) and by western blotting after 24 hr. (B) YM155 dose in (B) is 1μM. YM155 decreases Survivin expression at both the RNA and protein level. Data are representative of 3 experiments.**

To test the effects of Survivin antagonists on MB growth, we treated tumor cells with these agents and analyzed the percentage of cells expressing the proliferation marker Ki67. Consistent with our genetic results, inhibition of Survivin using either YM155 or S12 caused a significant decrease in the number of Ki67+ cells compared to treatment with vehicle (DMSO) (Figure 20A-D). Additionally, we saw a dose dependent decrease in thymidine incorporation after treatment with YM155, S12, or LLP3 (Figure 20E-F and Figure 22A). These data suggest that Survivin antagonists effectively inhibit MB growth *in vitro*.

To determine whether Survivin antagonists also cause cell cycle arrest in MB cells, we performed cell cycle analysis. ~~A~~After 24hrs, cells treated with S12 showed a significant accumulation in G2/M (56%) compared to cells treated with vehicle (12%) (Figure 21A,C). G2/M accumulation was also seen at 36h (52%) (Figure 21B,D). In contrast to S12, treatment with YM155 decreased the percentage of cells in G2/M (7%), with a concomitant increase in S phase (from 15 to 20% at 24hr) (Figure 21E-H).

Notwithstanding these differences, our data demonstrate that both Survivin antagonists alter normal cell cycle progression of MB cells.

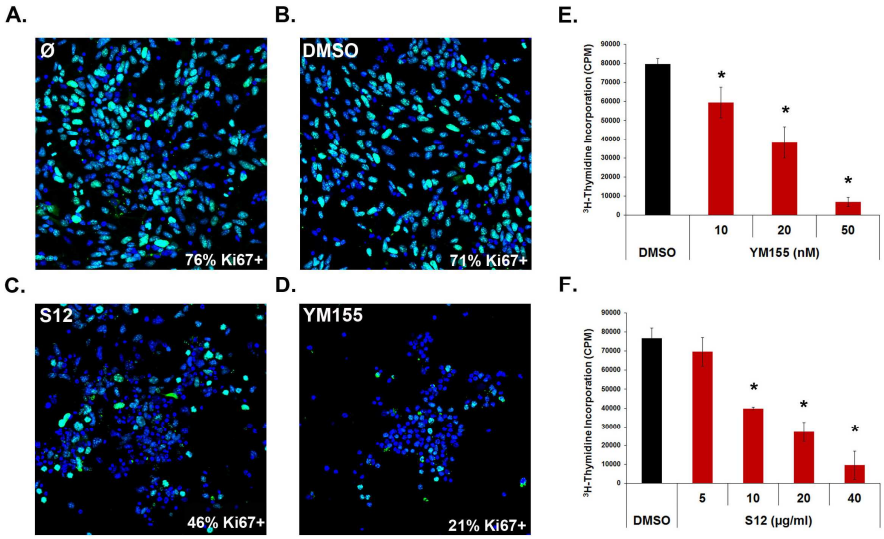

Figure 20: Survivin antagonists inhibit proliferation.

(A-D) Tumor cells were plated on chamber slides and treated for 24 hr with control media (A), 0.1% DMSO (B), 10 µg/ml S12 (C), or 50 nM YM155 (D). Cells were stained with anti-Ki67 antibodies (green) to mark proliferating cells and DAPI (blue) to label cell nuclei. Very few cells were Ki67+ after treatment with Survivin antagonists compared to controls ($p<0.0001$ for YM155 and S12). Data are representative of 3 experiments. (E-F) Ptch mutant tumor cells were treated with multiple doses of YM155 (E) or S12 (F) for 48 hr and pulsed with 3H-thymidine for 12 hr to measure proliferation. Treatment with either antagonist decreased proliferation in a dose-dependent manner ($p< 0.02$ for all YM155 and S12 doses except 5µg/ml S12, which was not significant (NS)). Ki67+ percentages in (A) were averaged from 6 images for each treatment. Data in (E-F) represent mean +/- SD and are representative of 6 experiments. Stats were calculated by ANOVA and post hoc student's t-tests.

In addition to its role in regulating cell cycle progression, Survivin has been suggested to function as an inhibitor of apoptosis. To determine if Survivin inhibition promotes MB cell apoptosis, tumor cells were treated with Survivin antagonists and then stained with Annexin-V and propidium iodide (PI). As shown in Figure 21E-G,

antagonists increased the percentage of apoptotic (AnnexinV+) tumor cells from 21% (after DMSO treatment) to 62% (S12) or 59% (YM155). Similarly, LLP3 treatment increased the percentage of Annexin+ and PI+ cells (Figure 22B-E). These data show that Survivin antagonists are not merely cytostatic, but can promote apoptosis of tumor cells as well.

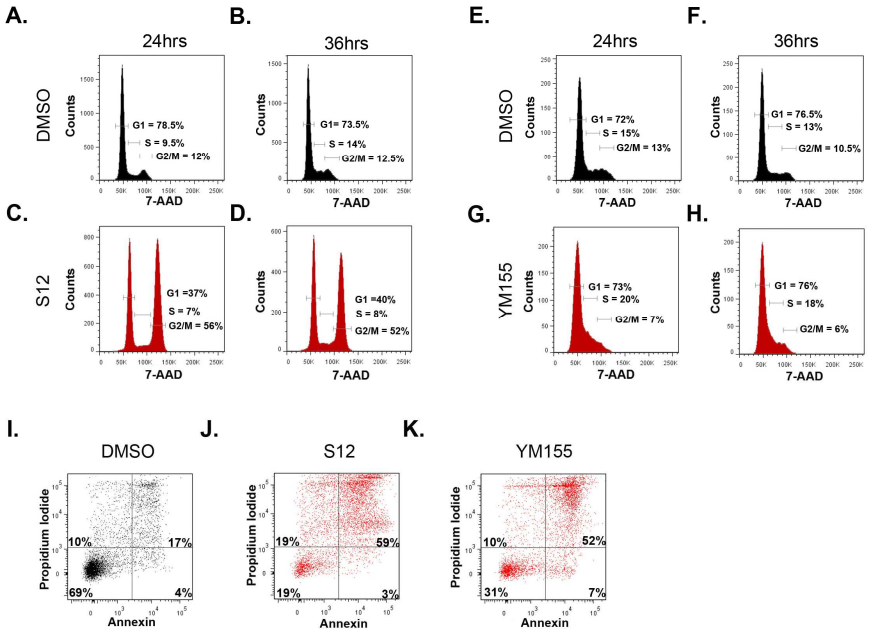

Figure 21: Survivin antagonists alter cell cycle progression and promote apoptosis.

(A-H) *Ptch* mutant tumor cells were treated with either DMSO (A,B,E,F), 20 µg/ml S12 (C,D), or 100 nM YM155 (G,H) and stained with 7-AAD for cell cycle analysis after 24 hr (A,C,E,G) or 36 hr (B,D,F,H). YM155 decreased the percentage of cells in G2/M, while S12 treatment caused an accumulation of cells in G2/M. Data represent 4 (A-D) and 6 (E-H) experiments and percentages based on live cell gates (excluded subG1). (I-K) Tumor cells were treated with DMSO (I), 20 µg/ml (J), or 100nM YM155 (K) for 36 hr, then collected and stained with Propidium Iodide (PI) and Annexin-V for FACS analysis. The percentage of apoptotic cells was significantly higher after antagonist treatment compared to control. Data represent 6 independent experiments.

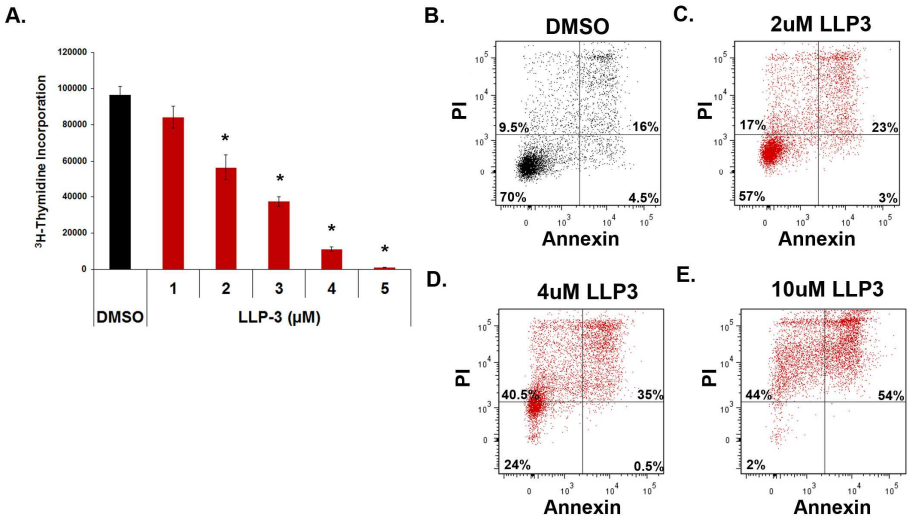

Figure 22: LLP3 inhibits proliferation and causes cell death of *Ptch* mutant tumor cells.

A) *Ptch* mutant tumor cells were treated with DMSO or multiple doses of LLP3 for 48 hr and pulsed with 3H-thymidine for 12 hr to measure proliferation. Treatment decreased proliferation in a dose-dependent manner ($p<0.05$ by ANOVA with post hoc student's ttest). Data represent mean +/- SD and are representative of 3 experiments. (B-E)Tumor cells were treated with DMSO (B), 2 μM (C), 4 μM (D), or 10 μM LLP3 (E) for 36 hr, then collected and stained with Propidium Iodide (PI) and Annexin-V for FACS analysis. The percentage of Annexin and PI+ cells was increased after treatment with LLP3. Data are representative of three experiments.

To verify that Survivin antagonists were not inducing non-specific toxicity, we tested their effects on GNPs, which express *survivin* (see Figure 16), and post-mitotic neurons, which do not. Treatment of GNPs with YM155 or S12 caused a dose dependent increase in the percentage of dead cells (as measured by EthD1 staining). In contrast, survival of post-mitotic neurons was not affected by treatment with Survivin antagonists (Fig 23A,B). These data suggest that Survivin antagonists induce death of Survivin-expressing cells but are not toxic to normal cerebellar neurons.

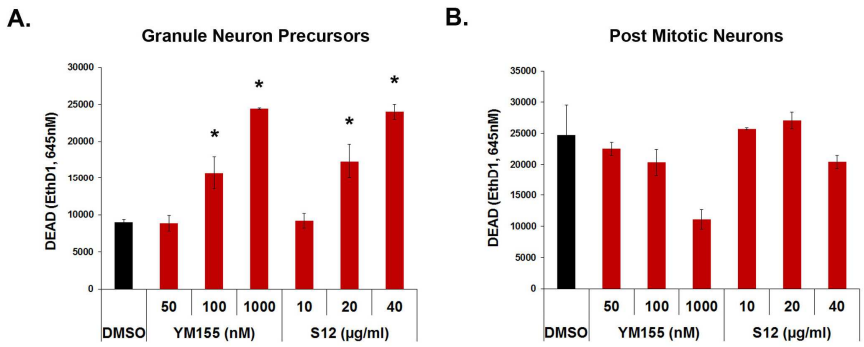

Figure 23: Survivin antagonists kill GNPs but not do not affect survival of post-mitotic neurons.

Granule Neuron Precursors (GNPs) were isolated from P7 wild type cerebella, split into two treatment groups, and plated in either SHH-containing media (A) or differentiation media (B). Cells in SHH-containing media were treated immediately with DMSO, YM155, or S12 for 48 hr and incubated with ethidium homodimer1 (EthD1) to mark dead/dying cells (A). Cells in differentiation media were cultured for 5 days, followed by treatment with DMSO, YM155, or S12 for 48 hr and incubation with EthD1 to mark dead/dying cells (B). Both S12 and YM155 caused GNP cell death at high doses ($p<0.01$ for high doses. NS for 50nM YM155 and 10µg/ml S12), but did not kill PMNs ($P>0.2$, NS for all doses). Data represent mean +/- SD and are representative of 4 independent experiments.

3.2.4 Survivin antagonists cooperate with radiation and SHH antagonists

Among the major drawbacks of current MB therapy are the devastating side effects of radiation[232,233]. Since recent studies have suggested that YM155 can enhance the efficacy of radiotherapy against NSCLC[234], we hypothesized that combining radiation and Survivin antagonists might be effective for MB as well. To test this, we performed thymidine incorporation assays on tumor cells treated with Survivin antagonists for 24hr followed by exposure to varying doses of radiation (Figure 24A). Treatment with sub-optimal doses of YM155 and S12 alone resulted in a small decrease in proliferation. The combination of Survivin antagonists and 0.25 grey (Gy) radiation markedly decreased

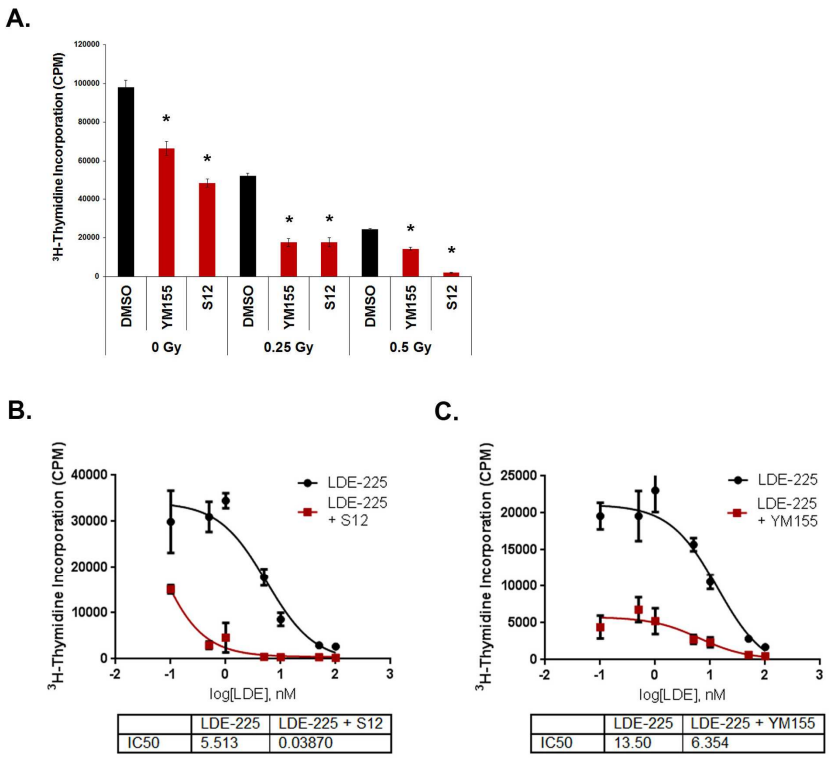

Figure 24: Survivin antagonists cooperate with radiation and LDE225 SHH antagonists.

A) Tumor cells were treated with either 0.1% DMSO, 50 nM YM155, or 10 µg/ml S12 for 24 hr, and then irradiated with 0, 0.25, or 0.5 gray (Gy). After 24 hr, cells were pulsed with ^{3}H-thymidine and assayed for incorporation. Treatment with antagonists enhanced sensitivity of tumor cells to radiation ($p<0.02$ for all treatments, calculated by 2 way ANOVA to identify radiation dose by treatment interaction, split by radiation dose, with post hoc student's t-tests) Data are representative of 5 experiments. (B,C) Tumor cells were plated in 96 well plates and treated with LDE225 alone (at the indicated concentrations) or in combination with 20 nM YM155 (B) or 10 µg/ml S12 (C). Cells were pulsed with ^{3}H-thymidine after 48 hr and harvested to assay levels of incorporation. Combination treatment of LDE with either S12 or YM155 significantly lowered the IC50 compared to LDE alone ($p< 0.01$ by student's t-test). Data are representative of 4 (B) and 3 (C) experiments. All data represent mean +/- SD.

tumor cell proliferation compared to radiation alone. The level of inhibition achieved with the combination treatment was equivalent to that achieved by doubling the radiation dose. These data suggest that Survivin antagonists can enhance the effects of radiation

on MB cells, and raise the possibility that these agents might allow reduction in the doses of radiation used for therapy.

In addition to conventional therapy, targeted therapies have begun to be evaluated for treatment MB. For SHH-associated tumors, NVP-LDE225 a small molecule antagonist of SMO, has shown some efficacy in animal models as well as in patients[21,235]. To test whether Survivin antagonists increase the efficacy of SHH antagonists, we treated *Ptch* mutant tumor cells with various concentrations of LDE225 alone or in combination with 10µg/ml S12 (Figure 5B). LDE225 alone had an IC_{50} of 5.5 nM (range: 3-8.5 nM) while the combination of LDE225 and S12 markedly decreased the IC_{50} to 0.04 nM (range: 0.04-2 nM). Similarly, exposure to 20 nM YM155 decreased the IC_{50} of LDE225 (from 13.5nM to 6.4nM). These data suggest that Survivin antagonists significantly enhance growth inhibition by SHH antagonists.

3.2.5 Survivin antagonists inhibit growth of human SHH-driven MB cells

The studies described above focused on murine SHH-associated MB. To determine whether human SHH-driven MB cells also respond to Survivin antagonists, we used patient-derived xenografts (PDXs) from SHH-driven tumors. Treatment of PDX cells with Survivin antagonists *in vitro* led to significant decreases in ^{3}H-thymidine incorporation (Figure 25A-C). Tumor cells were also treated with the SMO antagonist LDE225. Notably, the PDX line with a mutation upstream of SMO (DMB-012) was responsive to LDE225 [145,236] whereas the lines with mutations downstream of SMO (RCMB-018 and ICb-984MB) were resistant (Figure 25B,C, [237] and data not shown). In contrast, all three lines responded robustly to YM155 and high dose S12. These data suggest that Survivin antagonists can inhibit the growth of human SHH-driven MBs and that they may be useful for treating tumors that are resistant to SMO antagonists.

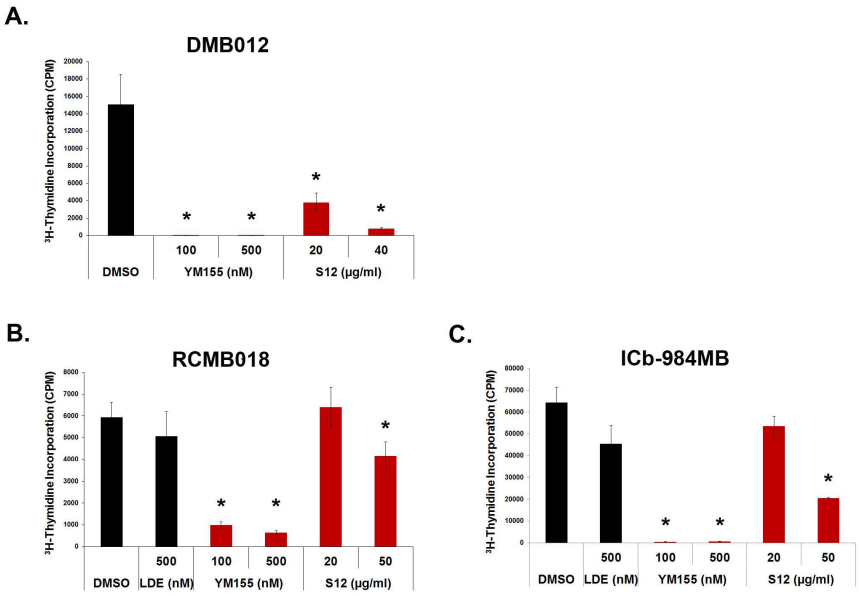

Figure 25: Survivin antagonists inhibit proliferation of human SHH-driven MB cells.

Cells isolated from LDE225-sensitive xenograft DMB012 (A) and LDE-insensitive xenografts RCMB018 (B) and ICb-984MB (C) were treated for 48 hr with DMSO, LDE225, YM155 or S12, and analyzed for thymidine incorporation following a 12-16 hr pulse. All tumor cells were sensitive to YM155 inhibition and high dose S12 treatment (DMB012 $p<0.01$ for all doses, RCM018 and ICb-984MB $p<0.03$ for YM155 and 10µg/ml S12 while LDE and 20µg/ml S12 were not significant with $p>0.08$). All stats were calculated by ANOVA and post hoc student's t-test. Data represent mean +/- SD and are representative of 3 experiments.

3.2.6 Non-SHH driven MB cells are also sensitive to Survivin inhibition

Seeing the efficacy of survivin antagonists against both LDE-responsive and resistance SHH driven tumors, we were interested to explore whether they may inhibit the growth of other subtypes of MB as well. Previous studies in our lab on a mouse model of Group 3 MB driven by Myc overexpression demonstrated that Survivin is highly expressed in these tumors compared to normal stem cells (the normal cell of origin for these tumors)[80]. Additionally, analysis of publically available databases of human MB gene expression revealed that BIRC5 is expressed in all 4 subtypes (Figure 26A). So

Survivin could be a viable target for these tumors. Treatment of either murine (MP)[80] or PDX group 3 MB cells with Survivin antagonists impeded proliferation as measured by ^{3}H-thymidine incorporation (Figure B-C). Finally, Group 4 PDX cells were also sensitive to Survivin inhibition by YM155 (Figure D). These data suggest that Survivin antagonists may be more broadly applicable for treatment of multiple subtypes of MB.

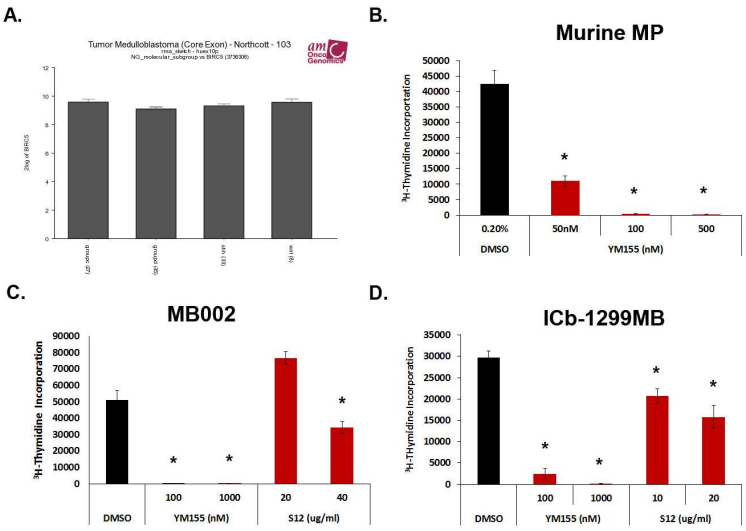

Figure 26: Non-SHH tumors express *survivin* respond to Survivin antagonists.

(A) R2 data from Northcott dataset was analyzed for *survivin* expression. All subtypes of human MB express *survivin*.(B-D) Cells were isolated from murine MP tumors (B), MB002 (group 3) xenograft (C), or ICb-1299 (Group 4) xenograft and treated with DMSO, YM155, or S12 (only C and D) for 48hrs. YM155 inhibits proliferation of both murine and human tumor cells. Both Group3 and Group4 tumors are sensitive to inhibition by S12 as well. (Murine MP, p<0.001, MB002, p<0.02 except for 20 µg/ml S12 which was NS, ICb-1299MB p<0.003 for all treatments). All stats were calculated using ANOVA and post hoc student's ttest. Data represent mean +/- SD and data from panels B-D are representative of two group tumors each.

3.2.7 Requirement of survivin for tumor initiation and maintenance

So far, both our genetic and pharmacological, have demonstrated the importance of Survivin for growth and survival of tumor cells *in vitro*. We were further interested in

the role of survivin *in vivo*. To test the importance of Survivin for tumor initiation, we used a genetic approach to delete Survivin at early stages of tumor formation. To this end, we crossed *Math1Cre;Ptch*$^{fl/fl}$ mice and *Survivin*$^{fl/fl}$ mice to generate the MCSP mice, in which *survivin* is deleted concurrently with *Ptch* in GNPs. We and others have previously used the *Math1Cre;Ptch*$^{fl/fl}$ mice to generate hedgehog-driven medulloblastoma[79,115]. These mice develop tumors with 100% penetrance and short latency of 6-12 weeks[115]. Although initially there seemed to be a delay in tumor latency when *survivin* is deleted, the survival curve shown in Figure 27 demonstrates that loss of *survivin* does not impair the ability of tumors to develop *in vivo.*

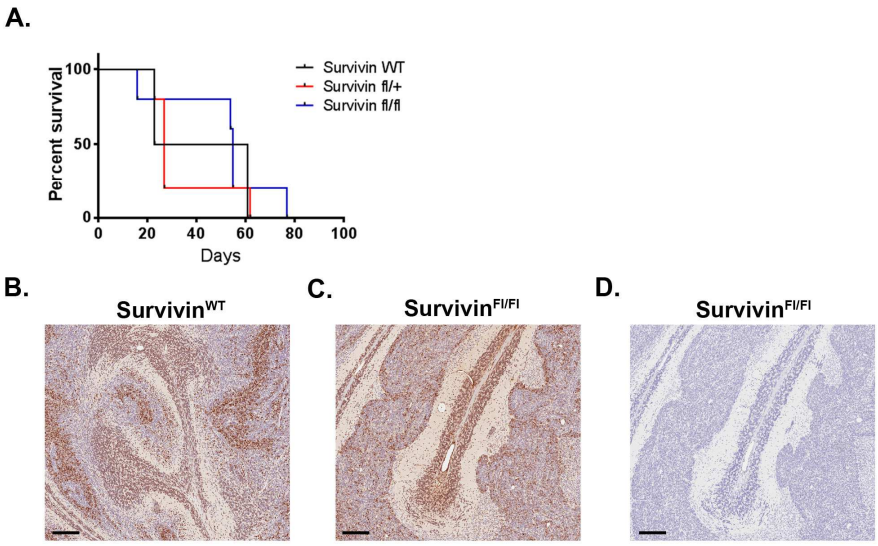

Figure 27: *In vivo* deletion of survivin may not inhibit tumor formation.

(A) Survival curve of Math1Cre; Survivin^{WT}, Survivin$^{fl/+}$, and Survivin$^{fl/fl}$ mice. All mice develop tumors with similar latency.(B-D) Tumors from Survivin^{WT} (B) and Survivin$^{fl/fl}$ (C,D) mice were stained with anti-survivin antibodies. All tumors displayed significant survivin staining (abrogated by blocking peptide (D)). Scale bars represent 100μM.

Staining of the resulting tumors with anti-survivin antibodies revealed that all tumors expressed Survivin, regardless of genotype (*Survivin*^{WT}, *Survivin*$^{fl/+}$(not shown),

or Survivin$^{fl/fl}$, Figure 27B-D). These data suggest that Survivin is either not required for tumor initiation, or more likely that tumors were able to form from cells that still express *survivin* due to insufficient deletion. Evaluation of *survivin* deletion in normal P8 GNPs (using *Math1CreER; Survivin$^{fl/fl}$* mice, Figure 28A) or deletion embryonically in *Math1Cre;Survivin$^{fl/fl}$* mice (Figure 28B-G) demonstrated that while there is some deletion of survivin *in vivo,* it may not be very efficient. This was surprising, as Math1Cre has been used to efficiently delete other genes in GNPs[24,115].

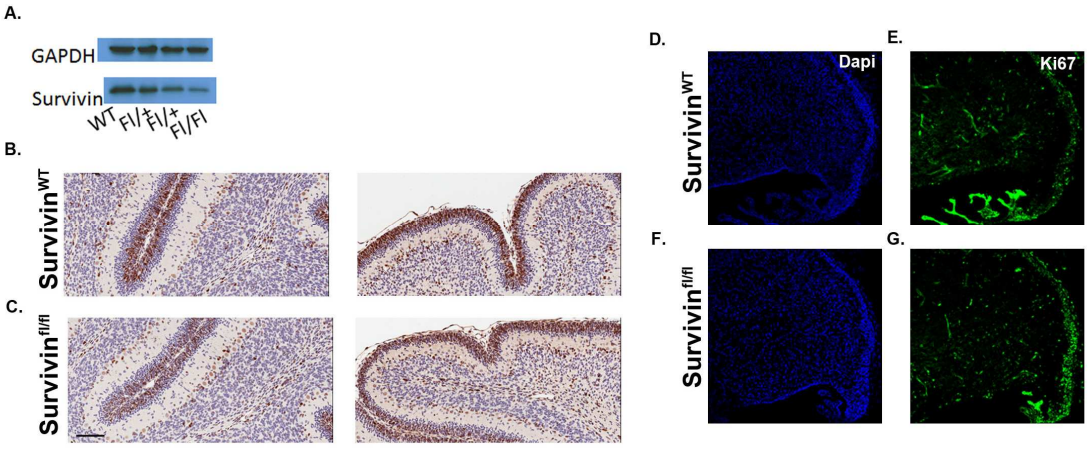

Figure 28: *In vivo survivin* deletion in GNPs is not efficient.

(A) *Math1CreER; Survivin^{WT,} Survivin$^{fl/+}$*, and *Survivin$^{fl/fl}$* mice were treated with tamoxifen at P4. Protein was isolated from P7 GNPs and survivin expression was analyzed by western blotting. *Survivin$^{fl/+}$* and *Survivin$^{fl/fl}$* cells displayed incomplete deletion, with slightly lower levels of Survivin than wildtype littermates. These data are representative of 4 experiments. (B,C) Cerebella from P7 *Math1Cre;Survivin^{WT}* (B) and *Math1Cre; Survivin$^{fl/fl}$* (C) were stained with anti-survivin antibodies to evaluate Survivin expression. Both *Survivin^{WT}* and *Survivin$^{fl/fl}$* mice express survivin in the EGL (where GNPs reside). Scale bars represent 50μM and data represent 2 experiments with 2 animals per condition. (D-G) Cerebella from E16.5 *Math1Cre;Survivin^{WT}*(D,E) and *Math1Cre;Survivin$^{fl/fl}$* (F,G) were stained with anti-Ki67 antibodies to mark proliferating cells (E,G) and Dapi to mark cell nuclei (D,F). *Survivin^{WT}* and *Survivin$^{fl/fl}$* cerebella display similar proliferation in the ventricular zone and EGL.

To analyze the ability of *survivin* deleted cells to form tumors *in vivo*, cells from SP mice were infected with Cre-IRES-GFP expressing viruses and sorted for GFP expression to select for *survivin* deleted cells. Once collected, these cells were transplanted into cerebella of immunocompromised mice and monitored for survival. We have demonstrated previously that *survivin* is deleted efficiently using this approach (see Figures 17A and 18B,C). Preliminary studies suggested that loss of survivin impedes tumor formation (Figure 29A-E), but subsequent studies with either viral Cre-mediated deletion or tamoxifen induced deletion in tumor cells derived from *Survivin$^{fl/fl}$; Math1-Cre-ER; Ptc$^{+/-}$ (SMEP) mice* were inconclusive due to variable tumor take after transplantation in controls (See Table 1).

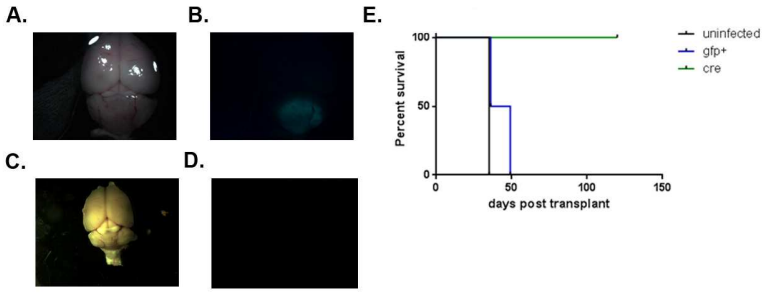

Figure 29: Preliminary Cre-mediated *survivin* deletion inhibits tumor formation.

SP tumor cells were infected for 36hrs with Cre or GFP expressing viruses, sorted, and GFP+ cells were transplanted into the cerebella of NSG mice. Whole mount (A,C) and GFP (B,D) images from control GFP-infected mice (A,B) and Cre infected mice (C,D). GFP infected cells formed GFP+ tumors, Cre infected cells did not form a tumor. (E) Survival curve of mice implanted with uninfected, GFP+, and Cre+ SP tumor cells. Five mice were analyzed in initial experiment.

Similarly, treatment of SMEP tumor-bearing NSGs *in vivo* with tamoxifen to determine importance of survivin for tumor maintenance were equally variable and thus difficult to interpret (data not shown). Despite our technical challenges in establishing the role of

survivin in *in vivo* tumors, it is possible that survivin is still critical for growth and maintenance of tumors and that targeting it would be effective.

Table 1: Variability in tumor take following GFP+ and Cre+ SP tumor transplantation or *in vitro* tamoxifen treatment of SMEP tumors

Experiment	Transplanted cells		
	GFP+	Cre+	GFP- control
1	2/2	0/1	2/2
2	1/1	1/5	1/4
3	0/2	0/1	-
4	1/2	0/2	1/2

Experiment	Treatment	
	DMSO	tamoxifen
1	2/4	1/3
2	1/2	1/2
3	2/3	2/4
4	2/3	1/4
5	4/5	4/5

3.2.8 Survivin antagonists do not inhibit growth of intracranial tumors due to poor blood brain barrier penetration

Given the ability of Survivin antagonists to inhibit proliferation and promote apoptosis of tumor cells *in vitro*, we questioned whether inhibiting Survivin could prevent tumor growth *in vivo*. To test this, MERP mice were treated with tamoxifen at P4 to delete *Ptch* and allowed to develop tumors for 3 months. Mice were then treated with YM155 or vehicle by microosmotic pump for 2 weeks and tumor volumes calculated. There was no detectable difference in tumor size between treated and control mice (Figure 30A-C), due in part to the large variability in starting tumor size between mice. Previous studies have shown that there is a spread in tumor latency from 12-19 weeks in these animals[115], so it is perhaps not surprising that within the cohort of mice there was a range of small and large tumors that obscured results.

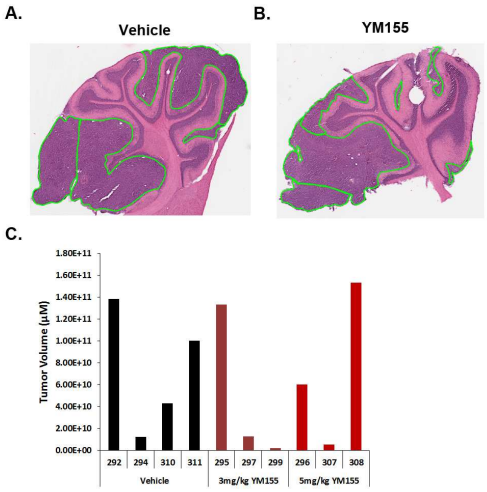

Figure 30: YM155 doe not inhibit the growth of endogenous *Ptch* mutant medulloblastomas.

Three month old MERP mice (tamoxifen treated at P4 to delete *Ptch*) were implanted with microosmotic pumps containing saline control (n=4), 3mg/kg YM155(n=3), or 5mg/kg (n=3) YM155 and treated for 2 weeks. Tumors from vehicle (A) and YM155 (B) treated mice were H&E stained and tumor volumes were calculated (C). Green boundaries indicate tumor areas counted. Tumors in both the vehicle and YM155 treated groups were variable in size.

To overcome the variability in tumor size encountered with endogenous tumors, *Ptch* tumor cells were isolated, infected with luciferase expressing viruses, and transplanted into the cerebella of NSG mice for treatment. Mice were grouped based on bioluminescence signal (as a surrogate for tumor size) and treated with either 5mg/kg/day YM155 (Figure 31A), 15mg/kg S12 (Figure 31B), or vehicle control. As can be seen in Figure 31, neither treatment resulted in significantly prolonged survival compared to vehicle treated controls, suggesting that our antagonists do not inhibit the growth of intracranial tumors. This was surprising, given the potency of these compounds *in vitro*.

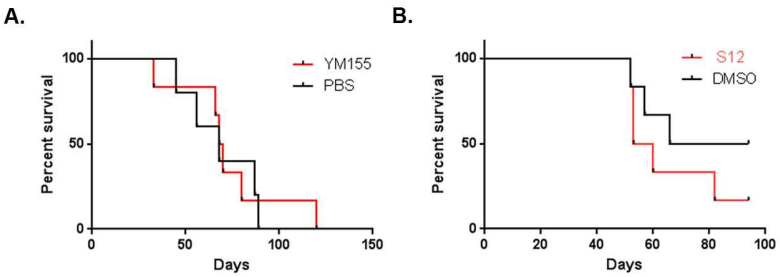

Figure 31: Survivin antagonists do not inhibit intracranial tumor growth.

***Ptch* mutant tumor cells were infected with luciferase expressing viruses overnight and transplanted into the cerebella of NSG mice. Mice were placed into treatment groups based on luciferase signal and treatment commenced after one month. (A) Mice were treated for 28days with 5mg/kg/day YM155 or saline control via microosmotic pump and monitored for survival.(n=6 YM155, n=5 vehicle) (B) Mice were treated with 15mg/kg S12 or vehicle 5x a week by i.p. injection and monitored for survival (n=5 YM155, n=4 DMSO). Neither YM155 or S12 were able to prolong survival. These data are representative of two experiments.**

A possible explanation is inability of these molecules to cross the blood brain barrier (BBB), a major obstacle for many potential CNS therapeutics[238]. To address this, we collaborated with the SBMRI Exploratory Pharmacology Core to perform an *in vitro* parallel artificial membrane permeability assay (PAMPA) assay to mimic BBB penetration. While LDE-225 was shown to be highly brain permeant, as expected, this assay demonstrated quite clearly that YM155 was unlikely to cross the BBB (Table 2). Indeed, a recent study from Minematsu et al. has demonstrated YM155 does not accumulate in the brain to any appreciable degree[239]. S12 on the other hand would be predicted to be able to cross at least moderately well (Table 2).

Table 2: *In vitro* blood brain barrier permeability of survivin antagonists

Compound	Avg. Pe (10-6 cm/s)	-log Pe
Verapamil-HCl	140	3.8
Corticosterone	15	4.8
Theophylline	0.27	6.6
LDE-225	278	3.6
YM155	1.4	5.9
S12	124	3.9

Despite the prediction that S12 should be able to cross the BBB, we did not see *in vivo* efficacy with S12. To address whether S12 accumulates at high enough levels *in vivo* to inhibit tumor growth, we performed pharmacokinetic studies in collaboration with the core at Sanford Burnham in Lake Nona. SCID/Beige mice were implanted with *Ptch* mutant tumors and tumors were allowed to develop for 5-6 weeks. Mice were then treated with a single dose of S12 (15 mg/kg) and plasma, normal brain, and tumor were harvested after either 30 mins or 2 hours. As can be seen in Table 3, while S12 does get in to the brain and tumor tissue to some degree initially (highest concentration in tumor was 1.1 µg/ml at 30mins), the antagonist is quickly cleared from the tumor tissue (average 0.163µg/ml). These levels do not even approach the *in vitro* IC50 for S12 (~10 µg/ml, see Figure 20F), so clearly the necessary levels of S12 are not being achieved in intracranial tumors. Similar rapid clearance was observed in the plasma of treated mice. Interestingly, normal brain tissue continued to accumulate S12 between 30 mins and 2 hours after treatment, although levels were also relatively low. These data together suggest that our antagonists do not accumulate in intracranial tumors enough to inhibit growth.

Table 3: PK of S12

Timepoint	S12 Dose (mg/kg)	plasma (ng/ml)	Brain (ng/g)	Tumor (ng/g)
30 min	15	268 +/- 42	416 +/- 394	828 +/- 288
2 hr	15	57 +/- 42	1066 +/- 250	163 +/- 144

***N=3 mice for each timepoint**

3.2.9 Survivin antagonists inhibit the growth of MB flank xenografts

Given that published reports (see Ref 239) as well as our own preliminary studies suggested that our Survivin antagonists do not accumulate in the brain or intracranial tumors sufficiently, we moved to a flank tumor model to test the ability of the antagonists to inhibit tumor growth *in vivo. Ptch* mutant tumors were implanted into the flanks of Nu/Nu mice and treated with YM155 or vehicle. Intratumoral injections of YM155 significantly decreased tumor growth compared to treatment with vehicle (Fig7A). Tumors harvested after 6 weeks of treatment were much smaller than those in the vehicle treated mice (Fig7B, C). We also tested whether systemic treatment with YM155 could inhibit tumor growth *in vivo*; since YM155 has a short half-life, we used osmotic pumps for delivery[216,229,240]. Nu/Nu mice bearing *Ptch* mutant flank tumors were implanted with micro-osmotic pumps to continuously infuse YM155 or vehicle (saline) for 3 weeks. Tumors in mice with YM155-containing pumps grew significantly less than those in mice with vehicle pumps (Fig 7D-F). These data suggest that Survivin antagonists can potently inhibit MB growth *in vivo*.

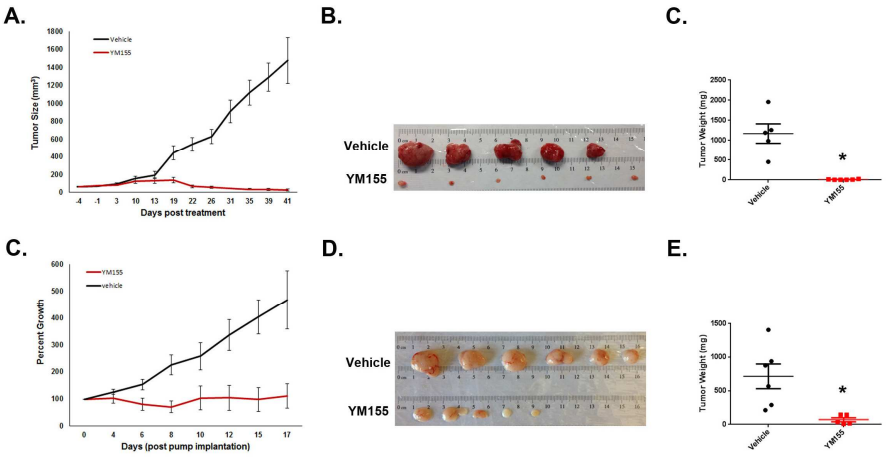

Figure 32: YM155 inhibits growth of Ptch mutant tumor cells *in vivo*.

(A-F) Tumor cells were suspended in GFR matrigel (1:1 with media) and implanted in the flanks of Nude mice. When tumors reached ~100mm^3, mice were split into two cohorts and treatment was started. For (A-C), tumors were treated with vehicle (20% DMSO in saline) or YM155 (20 μM) via intratumoral injection twice a week (vehicle n=5, YM155 n=6). For (D-F), mice were treated with vehicle (saline) or YM155 (10 mg/kg/day) via micro-osmotic pump (vehicle n=6, YM155 n=5). Caliper measurements were made twice a week to monitor tumor growth (A,D) and resulting tumors were collected, photographed (B,E), and weighed (C,F). Experiments were repeated 2 (A-C) and 3 times (D-F) respectively. Both intra-tumoral and systemic treatment with YM155 decreased tumor size over time compared to vehicle control. ($p<0.02$ for IT and pump YM155 tumor weight by ANOVA with post hoc student's t-test). Error bars represent SEM.

3.3. Discussion

Previous studies have shown that Survivin is expressed in human MB, and that its expression is correlated with poor outcome. To study the role of Survivin in MB growth and survival, we used a mouse model of MB driven by activation of the SHH pathway. Our studies demonstrate that Survivin is highly expressed in SHH-driven MB and that genetic and pharmacologic inhibition of Survivin impedes growth of MB cells *in vitro* and *in vivo*.

Our initial studies demonstrated that Survivin is expressed at high levels in *Ptch* mutant MB cells and not in normal adult cerebellum, suggesting that it might represent a cancer-selective target. To test the functional importance of Survivin in MB cells, we used Cre viruses to delete Survivin from *Survivin*$^{f/flx}$*;Ptch*$^{+/-}$ tumor cells. Loss of Survivin resulted in markedly decreased proliferation and caused arrest in the G2/M phases of the cell cycle. These results are consistent with the function of Survivin as a member of the chromosomal passenger complex, which is critical for alignment of chromosomes during metaphase and for successful cell cleavage[164,167]. In addition, previous studies have shown that loss of Survivin leads to aberrant mitosis, centrosome amplification and failed cytokinesis in a number of cancers, including glioma and cervical cancer[173,241]. Thus, in MB as in other tumors, Survivin seems to be critical for normal proliferation and cell cycle progression.

To evaluate Survivin's utility as a therapeutic target, we used small molecule antagonists. Consistent with our genetic studies, Survivin antagonists significantly decreased proliferation and altered cell cycle progression. Interestingly, while S12

treatment caused accumulation of cells in G2/M phase (similar to *survivin* deletion), YM155 caused cells to accumulate in S-phase. This observation is consistent with previous studies that demonstrated YM155 treatment can cause a loss of new DNA synthesis and concomitant stall in S phase[242]. This discrepancy between S12 and YM155 could be due to differences in the kinetics or degree of Survivin inhibition induced by these drugs. Alternatively, it could result from differences in the mechanisms by which the drugs act: whereas S12 binds directly to Survivin protein[230], YM155 inhibits *survivin* expression by disrupting ILF3/NF110 complexes[243,244] or Sp1 binding[245] at the *survivin* promoter. Thus, in addition to potently decreasing *survivin* expression (see Ref [229] and Figure 19), YM155 may also alter expression of other genes that regulate cell cycle progression (e.g. cyclin D1, p27) [246,247], and thereby cause arrest at earlier stages of the cycle. In other words, YM155 may function as a broader inhibitor of cell cycle than other Survivin antagonists. Nonetheless, the fact that genetic deletion and several small molecule inhibitors all interfere with cell cycle progression in *Ptch* mutant tumor cells strongly supports the notion that Survivin is required for this process in SHH-driven MB.

In addition to their effects on the cell cycle, Survivin antagonists also increased the percentage of MB cells undergoing apoptosis. It remains unclear whether loss of Survivin function causes apoptosis directly or as a consequence of cell cycle arrest. Analysis of Annexin+ cells after treatment with Survivin antagonists showed that apoptosis is not detectable until 36hr, whereas cell cycle inhibition is observed by 12-24hr. These results are consistent with the notion that apoptosis occurs secondary to cell cycle arrest. The fact that Survivin antagonists not only inhibit proliferation but also cause death of tumor cells may make them potent therapeutic agents for MB.

Importantly, we demonstrate that Survivin antagonists do not display non-specific neuronal toxicity, suggesting that they may be safe to use in the clinic. Additionally, our data show that higher doses of these drugs are required to inhibit the proliferation of survivin-expressing precursors in the cerebellum than are needed to interfere with tumor growth. This indicates that there could be a good therapeutic window for use of these drugs. This is important to establish, since recent studies have demonstrated that survivin has a role in some restricted adult tissues, including T lymphocytes, hematopoietic progenitors, and endothelial cells[157]. Despite this, early stage clinical trial data with YM155 and other survivin targeting methods support the safety of these approaches, as minimal toxicity was observed and most the serious symptoms observed (nephrotoxicity) were completely reversible[218,219,248]. Together these observations suggest that Survivin antagonists may be a safe and effective way to treat MB patients.

Previous studies of glioma and other cancers have suggested that targeting Survivin can enhance the effects of radiation and chemotherapy[186,216,234,249-253]. In agreement with these data, we found that Survivin antagonists significantly enhanced the sensitivity of MB cells to radiation. Combining low dose radiation (0.25 grey) with S12 or YM155 was as effective at inhibiting tumor growth as doubling the dose of radiation. These data suggest that addition of Survivin antagonists may allow children to be treated with lower doses of radiation without decreasing therapeutic benefits. The approach could markedly reduce treatment-related side effects and improve the long term quality of life of MB patients.

A major advance in treatment of SHH driven MB has been the development of SMO antagonists. However, one drawback of these agents is the swift acquisition of resistance to the drug and tumor recurrence. Our studies show that combining LDE225

and Survivin antagonists significantly enhanced inhibition of proliferation compared to either drug alone. Although in these studies we focused on co-treatment, it would be interesting to look at the ability of Survivin antagonists to overcome LDE225 resistance. In this context, it is notable that in non-small cell lung cancer, downregulation of Survivin by either siRNA or treatment with YM155 reverses Erlotinib resistance[254]. It is also important to note that LDE225 and other SHH antagonists work at the level of SMO, and are thus ineffective for patients with mutations downstream in the SHH pathway[145]. We show that human PDX cells with such downstream mutations are still sensitive to inhibition by Survivin antagonists. This suggests that treatment with Survivin antagonists could potentially compliment current targeted therapies to enhance response. In addition, our preliminary studies suggest that that YM155 can inhibit growth of non-SHH-associated MB cells, both murine and human. Although questions remain about the specificity of YM155 for Survivin, these studies raise the possibility that Survivin may be a therapeutic target for a broad spectrum of MB patients.

Numerous studies suggest a role for Survivin tumor formation and progression. Increased expression of *survivin* in preneoplastic lesions in colon cancer[255,256], prostatic intraepithelial neoplasia[257], and preliminary studies from our lab in MB (data not shown) suggests up-regulation of *survivin* is an early event in malignant transformation. A recent study in liver cancer showed elevated survivin expression in dysplastic liver nodules and further that Survivin activity is critical for cell survival during tumor initiation[207]. Additionally, Survivin expression is important for tumor progression from prostatic intraepithelial neoplasia to adenocarcinoma[206]. Consistent with these findings, elevated expression is correlated with increasing tumor grade in numerous cancers, including colorectal cancer[258]. Genetic deletion studies aimed at evaluating the

importance of survivin for MB tumor initiation revealed that tumors still form in *Survivin*$^{fl/fl}$ animals and furthermore that resultant tumors express high levels of Survivin. This could indicate incomplete deletion of *survivin* in precursor cells and a strong selective pressure for those cells that maintain *survivin* expression. It is possible that cells without Survivin stop proliferating and/or undergo apoptosis and tumors form from the remaining Survivin wildtype cells. Although we have observed efficient deletion of Survivin *in vitro* by either virally- expressed Cre or tamoxifen treatment (See Figure 18 and data not shown), initial *in vivo* studies in GNPs and tumors have suggested deletion may not be as efficient under the Math1 enhancer *in vivo*. Additionally, the broad window of tumor latency for *Survivin*^{WT} mice in combination with the relatively small number of tumors we obtained in these studies may also have obscured any differences in latency that would have arisen in a larger dataset. The fact that all tumors still expressed Survivin suggests that it could still be important for tumor initiation or growth and warrants further study.

Lastly, we have shown that YM155 can inhibit tumor growth *in vivo*, either by direct intratumoral injection or systemic administration. These data strongly suggest that targeting Survivin could be an effective approach for treating MB. A major challenge in treatment of brain tumors is the ability of drugs to cross the blood brain barrier (BBB). The BBB, which consists of capillary endothelial cells joined by tight junctions and surrounded by a thick basement membrane and astrocytic endfeet[259,260]**,** strictly regulates the entrance of the majority of small molecules into the central nervous system[261]. Additionally, many drugs that do manage to cross the barrier are substrates for multi-drug transporters (MDTs), which pump them out of the brain[262-264]. Because of this, even compounds that potently inhibit tumor growth *in vitro* may not reach high enough concentrations in the brain to achieve therapeutic effects. Unfortunately,

pharmacokinetic studies by our lab and others[239] suggest that the Survivin antagonists we have tested do not show significant accumulation in the brain or in intracranial tumors. Additionally, *in vitro* studies by Iwai et al. suggested that YM155 may be a substrate for P-glycoprotein MDR1[265], a ATP family transporter that is known to be a critical regulator of BBB permeability to drugs[266,267]. Thus, chemical modification of these agents, or alternative modes of delivery may be necessary to make these agents useful for treatment of MB and other brain tumors.

Although our experiments combining Survivin antagonists with adenosine agonists to facilitate BBB penetration were not successful (data not shown), other treatments modalities aimed at disrupting the barrier may prove more beneficial. Since studies have suggested YM155 is substrate for MDTs, co-administration with an MDT inhibitor such as Elacridar, which has been safely used in clinical trials[268] could be pursued. Another paradigm that has recently emerged in the preclinicial studies for treatment of glioma is disruption of the barrier using focused ultrasound with intravenous microbubble injection[269]. This approach has been used to deliver chemotherapeutics[270-274] , stem and immune cells[275,276], and even antibodies[277-279] into the brain, and disruption was shown to be focal, reversible, and safe[280-282]. Also, a recent study has suggested that treatment with hedgehog antagonists such as LDE255 could facilitate delivery of other molecules to the brain through disruption of the barrier itself[283]. Treatment *in vivo* with this combination may be particularly effective due to both the cooperation of these particular drugs (as our studies *in vitro* showed*)* and enhanced delivery of Survivin antagonists to the tumor. This would be a promising avenue for future exploration.

Other methods of delivery don't impair the barrier itself, but instead circumvent it to get drugs to the tumors. Global disruption of the barrier could have severe

consequences, as the barrier is essential for protecting neurons from systemic toxins and maintaining proper homeostasis[284]. More targeted strategies would therefore be preferable. One possible clinically available method to enhance delivery to the brain is to use convection-enhanced delivery (CED). This approach involves implantation of catheter(s) directly into the tumor mass or resected tumor bed for local delivery, bypassing the BBB[285]. Clinical trials have shown successful delivery of both cytotoxins and antibodies for treatment of glioma using CED [286,287]. Intrathecal delivery (delivery directly into the cerebro-spinal fluid via catheter in the spine), is another viable clinical option for delivery of Survivin antagonists. It is commonly used to deliver analegesics for management of both cancer and non-cancer related pain when more conservative therapies fail[288]. Additionally, it has been explored in clinical trials as an avenue for delivery of antibodies for treatment of brain tumors[289,290]. Other more preclinical avenues such as nanoparticle- (or stem cell -) mediated delivery could also be evaluated. Numerous studies have demonstrated the ability of drug loaded nanoparticles to home to tumors[291] and BBB penetrable nanoparticles have recently been developed[292].

Given the potency of survivin antagonists *in vitro* and effects of YM155 as a single agent on flank tumor growth, targeting Survivin in MB is a very promising strategy for treatment of this disease. Pursuing new methods of delivery will be a critical next step to moving these therapies forward to treating patients with MB.

4. Discussion

In these studies, we have used mouse models to study early stages of tumor development and test a novel therapeutic target for treatment of medulloblastoma. Some major questions and concepts that we consider in this remaining chapter are: 1) utility of mouse models for preclinical testing, 2) importance of defining and studying the cell of origin, 3) implications of Wnt pathway activation for novel therapies, 4) relevance of mitotic inhibitors as cancer therapy, 5) overcoming resistance using Survivin antagonists, and 6) considerations of the blood-tumor barrier in development of novel therapies for MB

4.1 Mice as models for human disease

In Chapters 2 and 3, we utilize mouse models to study the role of WNT signaling in cells of the developing cerebellum and to evaluate the efficacy of survivin antagonists to treat Shh-driven MB. Proof of efficacy and safety in animal models is a major criterion for moving drugs forward into clinical trials. Despite the weight these studies carry, a dismal proportion of compounds that look promising in mice translate to efficacy in the clinic[91,293]. These failures to predict efficacy stem from numerous factors. The major (obvious) fact is that mice are not people. There are differences in how drugs are distributed through the body and how they are metabolized. Differences in underlying genetics of mice are a huge factor as well. Even if mouse models are driven by similar mutations as found in human cells, there are species-specific differences in the role of genes in different cell types which can lead to presentation of different phenotypes and altered response to therapy[294]. The ability to predict is tied to how closely models reflect the complexity of human disease in terms of genetics, tumor cell composition,

microenvironment interactions, and response to therapy and often, models recapitulate only certain aspects of the disease.

Beyond the inherent differences between rodent models and humans, many times faults in study design lead to inaccurate conclusions about compound efficacy[295]. There is no standard for how preclinical studies are carried out and frequently they are not designed with sufficient numbers and clinically relevant endpoints[295]. In a telling study by Perrin et al, compounds that were previously reported to slow down progression of amyotrophic lateral sclerosis but failed in human trials were subjected to testing using more rigorous guidelines (in the same models). Interestingly, all of the drugs were not efficacious in the re-tests and more closely resembled the human studies[296]. Additionally, failure in clinical trials can also be caused by incorrect patient selection. For example, SHH antagonists are not efficacious in MBs that are not driven by hedgehog pathway activation[236]. These compounds would therefore fail in trials that did not take into account MB subtypes and assign patients accordingly. Many promising therapeutics can be rejected for lack of effect because the relevant patient subpopulation has not been identified.

It is also important to note that majority of reports claiming poor correlation of efficacy between mouse and human trials are based on analysis of models of human cell line xenografts that may not be very representative of endogenous human tumor growth and response. After long term selection in culture, the cell lines have lost many of features of the original tumors and certainly no longer reflect the clonal heterogeneity of human tumors[297]. Importantly, it is known that stromal and immune factors can influence tumor evolution and drug response. Tumors from these models are grown in a foreign environment (commonly the flank) and are propagated in immuno-compromised

animals, eliminating the ability to model any of these critical interactions. Recent development and application of genetically engineered mouse models (GEMMs) and patient-derived xenografts (PDXs) for preclinical testing have gone far to address many of these failings.

While they are far from perfect, mouse models provide valuable information that can influence the design of human trials. GEMMs are designed to closely mirror human tumor development, with relevant mutations expressed in corresponding cell types. Importantly, they provide an avenue to study early stages of disease, which is not possible in cell lines or even primary tumor samples. These studies provide insights into how disease develops and factors that influence progression. Pre-malignant stages in tumor models have been used to identify potential drivers in SHH-driven MB[77], pancreatic cancer[298,299], and colorectal cancer[298,300]. They are particularly useful for elucidating microenvironment- tumor cell interactions and identifying potential sensitivities that can be exploited for therapy. Patient derived xenografts (PDX) provide some unique advantages as well. They capable of modeling the diversity and heterogeneity that occurs in human tumors, which is useful for studying tumor biology and response to therapy. Importantly, direct transplantation of tumors into orthotopic sites has been shown to maintain the features and expression profiles of the original tumors during propagation[301 237]. And while they do not have the advantage of growing in native environment, they are derived directly from human tumors and orthotopic models mimic a normal microenvironment to some degree. Initial studies that progressively test standard of care therapeutics in more complex GEMMs and now in PDX models suggest that there is significantly enhanced translational accuracy of these models[301,302]. It

remains to be seen how well predictions of these models for novel therapies translate to clinical efficacy.

Additionally, these models allow for evaluation of PD/PK parameters to assist with study design for human trials. While drugs may be metabolized differently in mice and humans, they provide in an avenue to start to evaluate bioavailability of the drug in the target tissue, which is difficult to assess in human tumors[303]. Studies in preclinical models of MB and colorectal cancer demonstrated how small changes in exposure of tissues to GDC-0449 corresponded to a big change in anti-tumoral activity[304], which is critical for determining dosing for trials. *In vivo* models are also extremely helpful for identifying potential organ toxicities and drug interactions that would not be addressed in cell line based testing. Interestingly, recent studies in mice have shown that dose timing has an effect on development of resistance. Studies have using intermittent dosing of vemurafenib not only prolonged response compared to constant drug exposure, but actually saw some tumor regression when drug was withdrawn due to acquired drug-dependence[305]. These types of studies are critical for preclinical development of drugs and designing optimal dosing schedules.

What is needed now are ways to increase the predictive power of our current models and/or development of more representative models. With our increased understanding of cancer genome, we are much better equipped to develop genetic models that are driven by relevant genetics and more closely phenocopy human disease. Beyond that, there are two main areas in which we can improve the preclinical power: model selection and modifications to study design.

Model selection will be a key factor for improving outcomes. Prior to use in a preclinical setting, models should be rigorously tested to verify that they recapitulate the

human disease in terms of histology, gene expression, display consistent growth phenotypes, and very critically, should be verified that they mimic response to standard of care therapies[297,302]. Another major factor is asking the right type of question in the right model. PDX models are suitable for testing therapies that specifically impact tumor cell intrinsic properties and have been useful for identifying mechanisms of resistance[306-308] while GEMMs are particularly useful for testing drugs that target non-tumor cells or influence immune system responses (microenvironmental factors). The importance of model selection is highlighted in a study by Oliver et al. Using a well characterized GEM model of pancreatic ductal adenocarcinoma, researchers identified poor drug perfusion as a mechanism of gemcitabine resistance in pancreatic tumors and further that depleting stroma using a SMO antagonist enhances drug delivery. In contrast, subcutaneous human xenografts were responsive to gemcitabine and were well perfused[309]. In this case, the GEM models more accurately represent the response seen in humans to gemcitabine and studies performed in PDX models were misleading.

Changes in study design will be another major way of increasing the predictive power of mouse studies. For preclinical studies, factors in study design such as splitting litters, balancing for gender across groups, and statistical modeling to determine animal numbers should be done routinely. Guidelines such as these (mirroring those used in clinical trials) have been suggested to make preclinical studies more uniform and interpretable[310,311]. Additionally, it may be helpful to track genes and closely monitor disease phenotypes such as survival time; in models that harbor multiple copies of a gene, they can be inherited unevenly leading to changes in disease phenotype over time and potentially mask drug response[296]. It will also be essential to test therapeutics in multiple models to demonstrate the robustness of response. Defining appropriate

readouts that translate to clinical endpoints will be important as well. Overall survival is an ideal preclinical endpoint, but only really appropriate for GEMMs. For PDX tumors, especially subcutaneous models, recurrence or progression may be a better endpoint to consider.[297]

In terms of human trials, it will be essential to test drugs in the correct patient cohort in order to see efficacy. Identification of relevant patient populations will come both from analysis of patient derived expression profiles and subtyping based on mouse models. There has also been recent advent of "co-clinical trials" in which mouse studies are run in parallel with human trials. Since the mouse trials move faster, they can provide a sense of how well the therapies will work, pinpoint potential mechanisms of resistance, and identify biomarkers that can influence parameters in the ongoing and future trials[312]. A co-clinical trial in KRas mutant lung cancer was run to determine if MEK inhibitor increases efficacy of docetaxel, a current standard of care therapy. In the mouse model, researchers found that cancers with mutations in KRAS and Lkb1 are resistant to this combination therapy, which can now be validated in human samples in the current trial.[313]. This study highlights the strengths of this parallel approach. Integration of improvements in study design and continued development and selection of appropriate mouse models will continue to increase the predictive power of preclinical models.

4.2 Studying the Cell of Origin

The studies in Chapter 2 were aimed at identifying a putative cell of origin for WNT driven tumors. It is known that WNT signaling is critical for early specification and development of the cerebellum, but it is unclear what cells are later responsive to WNT

signaling and can ultimately be transformed by it to form MB. Our studies show that WNT signaling is mitogenic for cerebellar stem cells, but not GNPs. And critically, the differentiation defects that prolonged signaling causes suggest that a cooperating mutation may be required for tumors formation. Knowing which cells are capable of responding to WNT provides insights into the early stages of transformation and identifies potential vulnerabilities that can be targeted for therapy. Identification of the cell of origin is therefore of great interest to both researchers and clinicians.

A major question that is left to address is how to study the cell of origin. It is impossible to study early stages of tumor development directly in humans. Examination of expression profiles from primary tumor samples can provide a glimpse of what cell types may have arisen from. For example, it was long suspected that SHH-driven tumors arose from GNPs in the cerebellum due to similarities in expression of lineage-specific markers such as Math1 and elevated expression of components of the hedgehog signaling pathway, which is a critical regulator of GNP proliferation. And indeed, studies in mouse models of this subtype have verified that GNPs can serve as a putative cell of origin[34,79,115]. But comparison of expression profiles and differentiation status is not sufficient to determine cell of origin. For example, it has been proposed that basal-like breast cancer is derived from basal stem cells due to their similaries in phenotype[314]. Surprisingly, deletion of Brca1 (commonly altered in basal-like tumors) and p53 in luminal progenitors induced tumors that resemble human basal-like breast cancer, while tumors arising from deletion in basal stem cells did not phenocopy the human disease.

Investigation into the cell of origin is commonly done using genetically engineered mouse models. Advances in mouse genetics in recent years has allowed for exquisite lineage restricted and temporally controlled expression of oncogenes or loss of

tumor suppressors in an endogenous and developmentally relevant setting. These methods have been used to investigate the cell of origin in numerous cancers including basal cell carcinoma, lung, breast, and prostate cancers [315], in addition to study of early stages of tumor development and progression. Elegant lineage tracing studies using a mosaic labeling strategy to delineate contributions of difference cell populations to glioma formation identified oligodendrocyte precursor cells as a putative cell of origin for glioma[316]. Additionally, studies in intestinal and lung cancers have demonstrated that introduction of mutations into various cell populations have significantly different effects; APC deletion in stem cells causes adenomas, while activation in transit-amplifying cells rarely induces benign intestinal tumors[317,318] . In NSCLC, $KRas^{G12D}$ causes adenocarcinoma in AT2 cells (not in bronchioavleolar stem cells) [319], while mutations of Rb1 and p53 in neuroendocrine cells alone causes development of SCLC[320] . These studies demonstrate the importance of pairing certain mutations and cellular contexts in order to model specific subtypes of disease. Since mutations only cause transformation in certain cells, understanding the reasons for their particular sensitivity would be helpful in identifying new therapies.

These approaches have also been used to identify the cell of origin in the context of MB as well. Numerous studies have demonstrated that multiple cerebellar progenitor cells can form MBs after activation of the Shh pathway[34,79,115]. Additionally, a recent paper by Gilbertson et al identifies BLBP+ brain stem progenitors as a potential cell of origin for WNT-driven tumors[24]. They demonstrated that these tumors closely resemble the human WNT-associated disease in terms of tumor location in proximity to the brain stem and gene expression. Importantly, their data are in agreement with ours that WNT pathway activation alone is not mitogenic for GNPs of the cerebellum. While this study

provides evidence for a cell of origin for WNT tumors, this does not exclude the possibility that cells from the cerebellum are another source of tumor-generative cells. Their model was compared to a small subset of human tumors (n=6), so may only be representative of a small portion of the WNT subtype tumors. Unfortunately, due to the deleterious effects of WNT activation in cells outside the cerebellum, we were not able to follow the long term fate of cerebellar stem cells to determine if they represent a true cell of origin. Additionally, our finding that WNT activation causes self-renewal defects in our cells suggests that additional mutations may be required in these cells to support extended growth. Studies with more restricted Cre driver expression and coupled with introduction of cooperative second hits will be critical for addressing the cell of origin question for this subtype.

4.3 Target identification in WNT tumors

Whether or not we have identified the right cell of origin for these tumors, there are important implications of our studies for development of therapies for WNT tumors. We show that WNT signaling is mitogenic for stem cells initially, but more importantly that prolonged elevated signaling causes defects in self renewal. Since WNT driven tumors do occur, this suggests that in some contexts (cellular and/or genetic), WNT signaling can promote tumor formation. Whatever mechanism used to overcome this defect to allow for extended proliferation and progeny generation in tumors could represent a point of vulnerability for these tumors. Identifying the second hit that these cells depend on for growth and survival will therefore be critical for developing new therapies. To do this, analysis of human genomics coupled with mouse studies such as ours will be needed. In the case of the Gilbertson model, WNT activation cooperates

with p53 inhibition to cause accelerated tumor formation. This could be representative of the ~10% of human WNT-driven cases that display altered p53 expression. The remaining majority of cases are still unrepresented and need to be evaluated. Using our findings, closer analysis of human MB datasets with an eye towards genes that could influence stem cell fate determination may provide critical insights into cooperating mutations and development of a novel WNT-driven model that represents other subsets of WNT patients. The most common genetic alteration that occurs in this subgroup of tumors is monosomy 6, so genes that are expressed on this chromosome (e.g. p53) may represent cooperating second hits. Alternatively, a recent study has identified unique chromosomal modifiers that are mutated in some WNT driven MB tumors, including *SMARCA4* (ATPase) and *CREBBP* (histone acetyltransferase) which could represent novel targets[321].

4.4 Mitotic Inhibitors as targeted therapy

Our studies in Chapter 3 highlighted the efficacy of targeting Survivin, a key regulator of mitosis, for treatment of MB. Based on the idea that tumor cells proliferate more rapidly than normal cells, proteins that regulate the cell cycle are generally considered attractive candidates for selectively inhibiting tumor growth and sparing normal, less proliferative tissues. A number of chemotherapeutic agents, such as microtubule targeting agents paclitaxel and vincristine, have shown significant efficacy in patients[322], although they do display some myeloid and neuronal toxicities due to altered microtubule dynamics in non-dividing cells[323-325] and resistance eventually develops[322]. Prompted by these successes and a desire to limit inhibition to actively dividing cells, focus has shifted to inhibiting mitotic regulators. Mitosis is considered to

be the most fragile part of the cycle due to the complex changes that have to be strictly orchestrated through this phase, and perturbations lead to extended mitotic arrest through activation of the spindle assembly checkpoint and eventual signals for mitotic cell death[326,327]. Antagonists targeting mitotic entry (e.g cyclin dependent kinases, CDK1/2), mitotic kinases (e.g. aurora kinases, polo-like kinases) and kinesin spindle proteins (e.g. KPS, Eg5, CENP) have shown promise *in vitro* and in xenograft models [327-333] and numerous clinical trials have been initiated using these compounds[327,331]. Consistent with this, our data suggest that targeting mitosis through survivin inhibition is an effective way to treat MB and clinical trials with survivin antagonists have shown modest responses in other solid tumors[218,222,225,248]. Together, targeting mitosis appears to be a promising avenue for therapy.

Despite the apparent efficacy of these inhibitors in preclinical tests, there is debate as to whether targeting mitosis is the right strategy for cancer therapy. In general, preclinical efficacy has not translated to clinical success; a very low response rate has been seen across clinical trials in solid tumors for antagonists of the aurora and polo-like kinases, or mitotic kinesins as single agents[328,331]. This could be due in part to the limited expression and function of these targeted molecules in G2/M phase[178,334]. Therapies that rely on inhibition of these proteins will only inhibit the small fraction of cells that are in G2/M phase at the time of treatment and spare the rest. While this is desirable to limit toxicity, there is evidence from radiological studies that doubling time for human solid tumors *in vivo* can be well over 100 days, in contrast to 3-6 day range suggested by xenografted tumors or *in vitro*[331,335]. It is likely therefore that very few cells would be affected by antagonists unless they were given frequently and for long periods of time. Additionally, a recent study with Eg5 has demonstrated that point mutations in the

protein can cause drug resistance to develop[336]. And in general, resistance to anti-mitotics can occur through a mechanism call “mitotic slippage”, in which arrested cells escape the arrest without completing mitosis and either die or instead form viable multiploid cells which can display increased chromosome instability that can affect further promote malignant growth[327,337,338]. Lastly, if there is a population of quiescent cells within the tumors, they will not be affected by mitotic targeted therapies and could contribute to resistance and recurrence. A number of studies have identified cell populations within leukemias, so called tumor-propagating (TPCs) or cancer stem cells, which are quiescent[339,340] and may therefore prove to be refractory to mitotic-targeted therapies

In contrast to the leukemias, Dr. Rich and others have identified TPCs (marked by CD133+ expression) in human brain tumors that are not quiescent[341,342] and would therefore be less likely to be a barrier to mitotic therapy. Consistent with this, previous studies in mouse models of Shh-driven MB have identified a TPC population characterized by CD15 expression that are a highly proliferative[343,344]. Studies from our lab (and others) have shown that inhibitors of Aurora and Polo-like kinases inhibit the proliferation of these cells[236] and are effective at impairing tumor growth *in vivo*[236,345]. Importantly, we have seen similar results with our Survivin antagonists in our studies outlined in Chapter 3; Survivin antagonists effectively inhibit both bulk tumor growth and proliferation of CD15+ and CD15- cells *in vitro* (data not shown). So targeting mitotic regulators such as Survivin may still be an effective treatment strategy for MB.

Importantly, Survivin antagonists are not just mitotic inhibitors. Survivin expression has also been noted in tumors with low mitotic index[346], suggesting that expression is not merely a consequence of increased proliferation. And while Survivin

plays an important role in mitotic progression, survivin is not necessarily unilaterally involved in mitosis. In tumors, survivin is commonly expressed throughout interphase, as well as S phase[347], while its expression is restricted predominately to G2/M phase in most untransformed cells. This aberrant expression may reflect alternative functions for Survivin beyond its mitotic role in tumors. Critically, Survivin may represent a particular point of vulnerability for tumors. It is at the intersection of numerous pathways that facilitate malignant growth, influencing proliferation, survival, angiogenesis, and mediating therapy resistance. Combined with the low expression in normal tissues, these functions of survivin make it an ideal target for MB treatment.

4.5 Survivin antagonists to overcome resistance

Focus in recent years in cancer treatment is the concept of personalized medicine and the development of targeted therapies. These drugs are based on specific alterations that cancers depend on for growth and (ideally) are selective for tumor cells, so are significantly safer to patients than traditional chemotherapy. One of the earliest success stories in development of targeted therapies is Gleevec (Imatinib). Gleevec inhibits Bcr-Abl[348], an oncogenic fusion protein that is expressed in the majority of patients with chronic myelogenous leukemia (CML)[349], and its phenomenal success transformed CML into a treatable disease[350,351]. Following this success, there has been a flurry of targeted therapies in clinical trials for numerous cancers, including BRAF and MEK inhibitors for melanoma[86], anti-Her2 antibodies or inhibitors in breast cancer[87], and EGFR and ALK inhibitors in NSCLC[352,353]. In MB, a number of small molecule antagonists of Smoothened (a regulator of the SHH pathway) have been tested in the Shh-subtype of medulloblastoma (LDE-225, GDC-0449, IP1-926) and have shown

efficacy in mouse models and are in trials[90,92-94]. One of the major drawbacks of targeted therapy however has been the rapid development of resistance[95]. Investigations in MB, breast cancer, melanoma, and others have identified some common mechanisms of resistance. Mutations in the drug target itself or in downstream effectors of the targeted pathway occur frequently[86,87,352,354,355]. Additionally, activation of alternative pathways that circumvent the dependence on the original target have been identified[86,87,352,354]. PI3K pathway activation is one such evasive mechanism that occurs commonly across cancer types[87,95,356]. What is needed now is either a way to prevent resistance or a ways to treat resistant tumors..

Survivin antagonists may represent an important tool for treatment of resistant tumors. Studies in lung cancer have already demonstrated the utility of targeting survivin (by siRNA or YM155 treatment) for reversing Erlotinib resistance[254]. So treating with a combination of targeted agents, including survivin antagonists, may be helpful in preventing the development of resistance. As mentioned above, a common mechanism of resistance is activation of the PI3K pathway. Survivin is a downstream target of this pathway (through Akt)[357,358], so it is possible that inhibition of survivin could prevent some of the survival advantages incurred by PI3K pathway activity. It is well understood that Survivin is at the intersection of numerous pathways associated with malignant growth, so inhibition may disrupt other signals that mediate resistance as well.

Our *in vitro* data with combination treatment of LDE-255 and Survivin antagonists suggests that they can cooperate to inhibit tumor proliferation. Whether this combination prevents the development of tumor resistance *in vivo* or whether tumors that have become LDE resistant can be inhibited by survivin antagonists is an interesting question that remains to be studied. The optimal timing of treatments will be important to address

as well. Advantages of up-front combination of agents vs sequential treatment should be investigated. And as some studies have suggested that cycles of recovery should be included in treatment regimens to decrease the likelihood of resistance developing, proper combination treatment in this context will be interesting to explore.

4.6 The blood-tumor barrier and delivery of CNS therapeutics

Our data suggest that survivin antagonists robustly inhibit the growth of MB in the periphery, while they are incapable of inhibiting intracranial tumor growth. One reason for this may be poor blood brain barrier penetration of our antagonist. It is well established that in healthy brain, the BBB is critical for excluding the majority of molecules from entry into the brain, including (and especially) potential therapeutics[359,360]. Similarly, there is mounting evidence that that there are alterations in the BBB as consequence of brain tumor growth and that the compromised barrier, so called blood-tumor barrier (BTB), is more permissive than the BBB[238]. Patients with high grade brain tumors display contrast enhancement in MR images and CT scans[361-363] as well as an increased infiltration of plasma proteins[364]. Additionally, gliomas display physical alterations in capillary endothelial cells[365] that form the barrier and downregulation of tight junction proteins (e.g. claudins, Occludin, AQP4), causing junction disruption and increased edema[366 362,365,367,368]. Further, studies demonstrate that p-Glycoprotiens (the major drug efflux transporters in the BTB) have lower expression in metastastic BTB as well[369], although reports are less clear in primary glioma models[370,371]. These data together suggest that the BTB is less intact than the healthy BBB and therefore may not pose as strong an issue for drug delivery.

While there is definitive evidence for an altered BTB in context of brain tumors, it is unclear whether the increased leakiness is sufficient to enhance drug delivery to tumors. There is evidence that increased contrast enhancement does not necessarily correlate with increased drug delivery[372]. Even with impaired barrier integrity, it is unlikely drugs can penetrate sufficiently to inhibit the entire tumor. Models of primary or metastatic brain tumors show heterogeneous permeability of the barrier around the tumor bulk[363,373], with highest permeability around the tumor core and minimal change around the growing, more infiltrative edges[374]. This translates to variation in drug distribution to tumor areas, such that it is unlikely that drugs can achieve high enough concentrations to kill all tumor cells[375]. Significantly, a study by Salphati et al. demonstrated that a PI3K inhibitor is incapable of inhibiting growth of infiltrative gliomas, while a brain-permeant version inhibits growth of both infiltrative and non-infiltrative tumors[376]. These studies demonstrated both that BTB permeability varies between tumor types (infiltrative vs growth as a mass) and that drugs designed to cross the barrier are more effective than those that don't, even in the context of a compromised BTB.

As further evidence that the BTB remains an impediment for treatment, interference with BTB function enhances efficacy of drugs against tumor growth. Osmotic barrier disruption enhanced distribution and efficacy of antibodies and doxorubicin-immunoconjugates against glioma xenografts[, 377,378], suggesting that there was still BTB-mediated exclusion of these therapeutics. Similarly, pharmacological inhibition or knockout of drug efflux transporters (of which many drugs are substrates[238]) augments drug delivery and efficacy against brain tumors[379 380]. Finally, drug levels in tumors in peripheral tissues are significantly higher than found in brain tumors[375,381]. This translates to an obvious difference in drug efficacy in gliomas grown in the periphery

compared to brain that suggests the barrier is still an obstacle for treatment of CNS tumors[382,383]. Consistent with this, our data suggest that there is a relatively intact blood-tumor barrier that prevents our small molecules from crossing at sufficient levels into the brain (see Figures 30-32 and Table 3). Importantly, this is in the context of both endogenous tumors and in a transplantation setting, where there is already the question of compromised barrier due to the physical disruption involved in implanting the cells.

Beyond initial entry into brain, there are other factors influencing drug efficacy that have to be addressed for treatment. The extent to which the tumor cells themselves have functional multidrug transporters is a critical issue. Some studies suggest that gliomas and glioma-associated ECs from new tumor vasculature express p-glycoprotien at similar levels as other regions of brain[371,384]. And as mentioned above, alteration in p-glycoprotein expression does lead to enhanced drug accumulation in tumors. So even if drugs get through a compromised barrier, they may still be excluded from tumor cells by transporters. Interstitial fluid pressure in tumors can also have a substantial impact on drug penetration through the tumor even if they get through the barrier[385,386]. These issues should be taken into consideration as we move forward with development of new treatments for brain tumors.

The future of treatment of tumors in the CNS, whether primary or metastatic, will be highly dependent on enhancing delivery to the tumors, both through the BTB and increasing uptake into tumor cells. In the discussion of Chapter 3, we highlight some potential methods of circumventing the barrier as a way to increase drug delivery to tumors; by transient disruption of the barrier (e.g. osmotic perturbations, FUS, inhibition of MDTs), bypassing it completely (e.g. CED or intrathecal delivery), or facilitated entry such as with nanoparticle mediated delivery. Methods to transiently disrupt or impair

barrier function will be necessary in order for promising candidates, such as Survivin antagonists, to be translated to the clinic for treatment of MB.

5. Materials and Methods

5.1 Methods for Chapter 2

5.1.1 Mice

Catnb$^{lox(ex3)/+}$ mice[117], *Apc*$^{lox/lox}$ mice[387] and *Catnb*$^{lox(ex2\text{-}6)}$[71] mice have been described previously. *hGFAP-Cre*[118] mice and *hGFAP-green fluorescent protein* (hGFAP-GFP)[116] mice were from Jackson Laboratories, and Math1-Cre mice[79,115] were provided by David Rowitch at UCSF. All mice were maintained in the Cancer Center Isolation Facility at Duke University. All experiments were performed in accordance with national guidelines and regulations, and with the approval of the Duke University Animal Care and Use Committee.

5.1.2 Immunostaining

Tissue was harvested from embryos or neonates, fixed in 4% paraformaldehyde (PFA), equilibrated in 25% sucrose at 4℃ and frozen in Ti ssue-Tek OCT (Sakura Finetek, Torrance, CA, USA). For bromodeoxyuridine (BrdU) staining, pregnant females were injected with 100 mg/kg BrdU prior to embryo harvest. Cryosections (12 □m) were blocked with 10% goat serum in 0.1% Triton X-100 at room temperature and primary antibodies were applied overnight at 4℃. Antibodie s included rabbit anti-Ki67 (Abcam, Cambridge, MA, USA; 1:100) or mouse anti- Ki67 (BD Biosciences, San Jose, CA, USA; 1:100), rabbit anti-Sox1 (gift of Larysa Pevny, University of North Carolina, Chapel Hill; 1:1500), rabbit anti-β-catenin (Abcam; 1:2000), rabbit anti-calbindin (EpitMics, Burlingame, CA, USA; 1:250), rabbit anti-Pax2 (Covance, Princeton, NJ, USA; 1:250), rabbit anti-Pax6 (Covance; 1:300), mouse anti-S100β (Sigma; 1:500), rabbit anti-Map2

(Chemicon/Millipore, Temecula, CA, USA; 1:1000), rabbit anti-BLBP (Chemicon; 1:500), mouse anti-GFAP [cocktail of monoclonal antibodies (clones 4A11, 1B4, 2E1), BD Pharmingen; 1:100] and rat anti-BrdU (Abcam; 1:100). Secondary antibodies included goat anti-mouse Alexa Fluor 594 and goat anti-rabbit Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) used at 1:200. Noggin, Bmp2 and Bmp7 proteins were purchased from Peprotech (Rocky Hill, NJ, USA) and used at 200 ng/ml. p21shRNA retroviral plasmid (Fasano et al., 2007) was a gift from Sally Temple at the Neural Stem Cell Institute, Rensselaer, NY, USA. Cre retrovirus was a gift from Tannishtha Reya (University of California, San Diego).

5.1.3 Cell isolation and flow cytometry

Cells were isolated from embryonic (E14.5) or neonatal (P0) cerebellum as described[78]. Briefly, tissue was digested in a solution containing 10 units/ml papain (Worthington) and 250 U/ml DNase, and triturated to obtain a single-cell suspension.To isolate prominin 1+ lineage-negative (Prom1+ Lin–) cells from wildtype (WT) or mutant mice, cells were suspended in FACS buffer (Dulbecco's PBS with 5% FCS). Cells were stained for 1 hour with phycoerythrin (PE)-conjugated rat anti-prominin 1 antibody (clone 13A4, eBioscience, San Diego, CA, USA; 1:200) and with antibodies specific for lineage markers [polysialylated neuronal cell adhesion molecule (PSA-NCAM), Chemicon/Millipore, 1:200; O4, Chemicon/Millipore, 1:100; TAPA-1 (CD81), eBioscience, 1:250]. After staining with FITC-conjugated secondary antibodies, cells were washed and analyzed or sorted using a FACS-Vantage SE flow cytometer (BD Biosciences). PE conjugated rat IgG was used as a negative control.

5.1.4 *In vitro* analysis of proliferation, differentiation and neurosphere formation

5.1.4.1 Ki67 Staining

To analyze the effects of β-catenin on proliferation, cells were cultured in Neurobasal medium with B27 supplement (NB-B27, Invitrogen) on poly- D-lysine (PDL)-coated chamber slides (5 x 10^4 cells/well). β-catenin-IRES-YFP or control YFP viruses were added and cells incubated for 48 hours before staining with anti-Ki67.

5.1.4.2 Thymidine incorporation

To assay incorporation of tritiated thymidine, cells were cultured in PDL-coated 96-well plates at 2 x 10^5 cells/well. β-catenin-IRES-GFP or control GFP viruses were added and cells incubated for 48 hours before being pulsed with methyl- [3H]thymidine (GE Healthcare, Piscataway, NJ, USA). After 16 hours, cells were harvested using a Mach IIIM manual harvester 96 (Tomtec, Hamden, CT, USA), and incorporated radioactivity was quantitated using a Wallac MicroB microplate scintillation counter (Perkin Elmer, Waltham, MA, USA).

5.1.4.3 Differentiation

To analyze the differentiation potential of WT or mutant NSCs, Prom1+ Lin– cells were plated on PDL-coated coverslips in NB-B27 plus 1% FBS, and cultured for 3 days before fixation with 4% PFA, permeabilization with 0.1% Triton X-100, and blocking with 10% goat serum. Cells were stained with anti-Map2 and anti-S100β.

5.1.4.4 Neurosphere formation

To measure neurosphere formation, Prom1+ Lin– cells were cultured at 2000 cells/ml in uncoated wells containing Neurocult with proliferation supplement (Stem Cell Technologies, Vancouver, BC, Canada) plus 25 ng/ml bFGF (Invitrogen) and 25 ng/ml

EGF (Peprotech). Cells were cultured for 7 days and photographed under bright-field microscopy. To examine self-renewal, spheres were dissociated in 0.05% trypsin/EDTA (Invitrogen) and replated at 2000 cells/ml.

5.1.5 Real-time RT-PCR

mRNA was isolated using the RNAqueous kit (Ambion, Austin, TX, USA) and treated with DNA-free DNase (Ambion). cDNA was synthesized using oligo(dT) and Superscript II reverse transcriptase (Invitrogen). Real-time RT-QPCR reactions were performed in triplicate using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and the Bio-Rad iQ5 Multicolor Real-Time PCR Detection System. Gene expression was normalized to actin (*Actb*), and expression of each gene was compared between WT and G-Cat mice from the same litter. Primer sequences are listed in Table 4.

5.1.6 Statistical analysis

Statistical significance was determined by Student's *t*-tests and, in the case of gene expression, by performing a two-factor ANOVA to identify genotype-by-gene interaction and post-hoc two-tailed *t*-tests (for *P*-value).

Table 4:Primers for qPCR

Gene	Primer	Sequence
Actin	Forward	TATTGGCAACGAGCGGTTCC
	Reverse	GGCATAGAGGTCTTTACGGATGTC
Bmi1	Forward	CAACTTCTCCTCGGTCTTCA
	Reverse	AGCTGATGCTGCCAATGGCTCCA
Hes1	Forward	GCCAATTTGCCTTTCTCATC
	Reverse	ACATGGAGTCCGAAGTGAGC

Hes5	Forward	CCTCACCTCAAGGTCCACAT
	Reverse	TGTTCTCCCACATGACCAAG
c-Myc	Forward	CTCTCCTTCCTCGGACTCG
	Reverse	GGTTTGCCTCTTCTCCACAG
Bmp2	Forward	TGGACGTGCCCCCTAGTGCT
	Reverse	GGATGCCGCGGCGAACTTCT
Bmp7	Forward	GCGCAGCCAGAATCGCTCCA
	Reverse	ATGGCGTGGTTGGTGGCGTT
p21	Forward	CTGTCTTGCACTCTGGTGTCTGAG
	Reverse	TTTTCTCTTGCAGAAGACCAATCTG

5.2 Methods for Chapter 3

5.2.1 Mice

Survivin$^{fl/fl}$ [160], *Ptc*$^{fl/fl}$ [388], *Math1-Cre-ER*[28], *Math1Cre* and *Math1CreER;ptc*$^{fl/fl}$ [115] (MERP) mice have been described previously. P4 MERP pups were gavaged with 0.8g/40µl of tamoxifen (T-5648, Sigma, St. Louis, MO) in corn oil to generate tumors. Tumors from *Ptch*$^{+/-}$ and MERP mice were used for experiments and are referred to as *Ptch* mutant tumors in the text. To allow for deletion of *survivin* in tumor cells, *Survivin*$^{fl/fl}$ mice were crossed with *Ptch*$^{+/-}$ mice to generate the *Survivin* $^{fl/fl}$*; Ptch*$^{+/-}$ (SP) line. This line was further crossed to Math1-Cre-ER mice to generate the *Survivin*$^{fl/fl}$*; Math1-Cre-ER; Ptc*$^{+/-}$ (SMEP) mice. To assess the effects of survivin loss on tumor formation, Math1Cre mice were crossed *with Survivin*$^{fl/fl}$ and *Ptc*$^{fl/fl}$ mice to generate the *Math1Cre; Survivin*$^{fl/fl}$*; Ptc*$^{fl/fl}$ (MCSP) line. MP tumor mice were described previously[80]. CD-1 Nu/Nu

mice and Fox Chase SCID/Beige (CB17.Cg-*Prkdc*^{scid}*Lyst*$^{bg-J}$/Crl) mice were obtained from Charles River Laboratories (Wilmington, MA). P7 wild type C57BL/6 pups were obtained from the SBMRI Animal Facility. All mice were maintained in the Animal Facility, and experiments were performed in accordance with national guidelines and regulations, and with the approval of the SBMRI Institutional Animal Care and Use Committee.

5.2.2 Cell isolation and *in vitro* culture

GNPs were isolated from P7 cerebellum and tumors from adult cerebellum as previously described[78,115]. Briefly, tissue was digested in a solution containing 10 U/ml papain (Worthington Biochemical Corporation, Lakewood, NJ) and 250 U/ml DNase (Sigma), and triturated to obtain a single-cell suspension. Cells were spun through a 35-65% Percoll gradient (GE Healthcare Uppsala, Sweden) to purify GNPs and tumor cells. Cells were cultured in NB/NS-21 media (Neurobasal media, 1 mM sodium pyruvate, 2 mM L-glut, penicillin/streptomycin and NS-21 supplement, plus 1% FBS (Invitrogen Grand Island, NY)) on growth factor-reduced (GFR) matrigel-coated plates (1:50 in NB/NS-21, BD Biosciences, San Diego, CA).

5.2.3 Real-Time PCR

For analysis of *survivin* expression, mRNA was isolated from cells and tissues using an RNAeasy Plus Mini kit (QIAGEN Inc, Valencia, CA). One-step qRT-QPCR reactions were performed in triplicate using QuantiTech RT mix (QIAGEN) on the Bio-Rad C1000 Thermocycler and CFX96 system (Bio-Rad Laboratories, Hercules, CA). Duplicate reactions were prepared without reverse transcriptase to confirm the absence

of genomic DNA contamination. Relative gene expression was calculated using the ΔΔCT method and normalized to Actin. 95% confidence intervals for each sample were calculated using the sum of the squares method. To evaluate the efficiency of *survivin* deletion by Cre infection, SP tumor cells were isolated, infected with Cre-IRES-GFP or GFP retroviruses for 48hrs, sorted for GFP expression, and analyzed as outlined above.

5.2.4 Immunohistochemistry

5.2.4.1 Survivin staining

For staining of paraffin-embedded tissue, animals were perfused with PBS followed by 4% paraformaldehyde (PFA, Affymetrix, cat# 19943). Cerebella and tumors were removed, fixed in 4% PFA overnight and delivered to the SBMRI Histology Shared Resource for embedding, antigen retrieval, and staining. Sections were stained either with anti-Survivin antibodies (Cell Signaling Technology Cat#2808S, Danvers, MA) alone or anti-Survivin antibodies pre-incubated with Survivin blocking peptides (Cell Signaling Technology Cat #1037).

5.2.4.2 Frozen section staining

For staining of frozen tissue, animals were perfused with PBS followed by 4% paraformaldehyde (PFA)(Affymetrix). Cerebella were removed, fixed in 4% PFA overnight, cryoprotected in 30% sucrose, frozen in TissueTek-OCT (Sakura Finetek, Torrance, CA), and cut into 10–12 mm sagittal sections. Sections were rehydrated in PBS (Invitrogen) for. Slides were then blocked and permeabilized for 1 hr with PBS containing 0.1% Triton X-100 (Aqua Solutions Deer Park, TX) and 1% normal goat serum (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) and stained overnight with anti-Ki67 (1:100 BD Pharmingen, San Jose,CA) or CC3 (1:200 Cell

Signaling Technology, Danvers, MA antibodies) at 4^0C. and incubated with alexa fluor rabIgG-594 secondary antibodies(1:200, Invitrogen Molecular probes Eugene, OR) for 2 hr at room temperature. Sections were counterstained with DAPI and mounted with Fluoromount-G (Southern Biotech, Birmingham, AL) before being visualized using a Zeiss LSM-700 confocal microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY).

5.2.5 Cell Lysis and Western-blotting

To evaluate Survivin expression, tumor cells, GNPs and adult cerebellum were lysed in RIPA buffer (Millipore, Billerica, MA). Protein was quantitated using the Bio-Rad protein assay. Equal amounts of protein were separated by SDS-PAGE, blocked with 5% BSA (Sigma) in Tris-buffered saline with 0.1% Tween-20 (TBST), and stained with anti-Survivin or GAPDH antibodies overnight (1:1000, Cell Signaling Technology Cat# 2808S, 5174) followed by anti-rabbit HRP-conjugated secondary antibody (1:2000 Cell Signaling technology, Cat# 7074S). Proteins were visualized by incubating with Pierce ECL plus (Thermo Fisher Scientific, Rockford, IL). To evaluate Survivin expression after YM155 treatment, tumor cells were plated on 6-well plates (6-8M cells/well) and treated with YM155 or DMSO (Fisher Scientific Inc. San Diego, CA) at 1 µM for 24hrs and processed as described above.

5.2.6 Analysis of proliferation, cell cycle and apoptosis

To analyze the effects of Survivin loss, cells were isolated from SP tumors and infected with Cre-IRES-GFP or GFP retroviruses (MSCV, 1:5 in media). To assess the effects of pharmacological inhibition of Survivin, *Ptch* mutant tumor cells were treated

with YM155 (Selleck Chemicals), S12[230], LLP3[231], or DMSO (Fisher Scientific) at the indicated concentrations.

5.2.6.1 Ki67 staining:

Cells were plated on GFR matrigel-coated chamber slides at 0.2M cells/well and treated with DMSO or antagonists for 24hrs. Cells were fixed in 4% PFA, permeabilized with 0.1% Triton X-100 in PBS (Aqua Solutions Deer Park, TX) and blocked with 10% goat serum (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) before staining with anti-Ki67 (BD Biosciences Cat# 556003) and 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). Six representative images from each treatment were collected using the Zeiss LSM-700 confocal and Ki67+ percentages calculated using ImageJ software (NIH).

5.2.6.2 Thymidine incorporation

Cells were plated (2 x 10^5 cells/well) in GFR-matrigel coated 96 well plates and treated with either virus or antagonists for 48hrs in triplicate wells before being pulsed with methyl-[^{3}H]thymidine (GE Healthcare, Piscataway, NJ, USA). After 12-16 hr, cells were harvested using a Mach III manual harvester 96 (Tomtec, Hamden, CT, USA), and incorporated radioactivity was quantitated using a Wallac MicroB microplate scintillation counter (Perkin Elmer, Waltham, MA, USA).

5.2.6.3 Cell cycle analysis

Cells were plated in GFR-matrigel coated 48 well plates at 0.4M cells/well, infected with virus or treated with antagonists, and collected at various time points. Cells were fixed and stained using the FITC BrdU Flow Kit (BD Biosciences) and 7-Aminoactinomycin (7-AAD) according to the manufacturer's instructions. Analysis was

performed using a FACSCanto II flow cytometer (BD Biosciences) and FlowJo v.7.6.4 software (Tree Star, Inc., Ashland, OR).

5.2.6.4 Apoptosis

Tumor cells were plated on 24-well plates at 1M cells/well and treated with Survivin antagonists or infected with viruses for 36 hr. Cells were collected by incubating with papain solution and resuspended in 100 ul of Annexin-binding buffer containing 5µL of AnnexinV conjugate (Annexin-FITC or Annexin-567, both Invitrogen) and 1µl Propidium iodide (PI, 1.0mg/ml stock, Invitrogen). Cells were analyzed using a FACSCanto II and FlowJo v.7.6.4 software.

5.2.6.5 Live/Dead Assay

To address toxicity of YM155, GNPs were isolated from WT P7 pups and plated at 0.2 x 10^6 cells/well in 2 96 well plates coated with GFR-matrigel for each experiment. One plate was maintained in proliferation media consisting of NB/NS-21 and Sonic hedgehog (SHH)-containing supernatant (1:5 in media) and treated with either DMSO or various doses of YM155 and S12 with each condition in triplicate wells. After 48hrs, cell viability was analyzed using the LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen). Briefly, cells were stained with 4µM EthD1 for 40 min and fluorescence emission (645 nM) was measured using a TECAN infiniteM200 Microplate reader (Morrisville, NC). The second plate was maintained in differentiation media (NB/NS-21 containing 25mM glucose and 25mM potassium chloride) for 5 days to produce post-mitotic neurons. Cells were then treated with DMSO or corresponding doses of YM155 and cell viability was evaluated after 48hrs.

5.2.7 Radiation and LDE225 treatment

To measure effects of inhibitors in combination with radiation, tumor cells were plated in 96-well plates at a density of 0.2 x 10^6 cells per well and cultured in the presence of DMSO, 50 nM YM155, or 10 ug/ml S12. After 24hrs, cells were subjected to 0, 0.25, or 0.5 Gy radiation using a Gammacell 40 Exactor (Low-dose cesium 137 irradiator, Best Theratronics Ltd., Ottawa, Ontario, Canada). Cells were cultured for an additional 24 hr, and [methyl-^{3}H]thymidine assays were performed as described above.

To measure effects of inhibitors in combination with the SHH antagonist NVP-LDE225 (Selleck Chemicals, S2151), tumor cells were plated in 96 well plates at 0.2 x 10^6 cells/well and cultured with increasing doses of LDE225 or a single dose of Survivin antagonist (10µg/ml S12, 20nM YM255) alone or in combination with LDE225 as indicated. Cells were cultured for 48hrs and [methyl-^{3}H]thymidine assays were performed as described above.

5.2.8 Human tumor isolation, propagation, and classification

Human MB tissue for patient-derived xenografts was obtained from surgical resection of tumors at Duke University Medical Center (Durham, NC), Rady Children's Hospital (San Diego, CA) or Texas Children's Cancer Center (Houston, TX). All procedures using human tissue were approved by the Institutional Review Boards of the respective institutions. Upon retrieval, the tissue was mechanically dissociated into a single-cell suspension, then immediately injected into the cerebella of NSG mice. When mice showed signs of MB, tumors were again dissociated into single-cell suspensions and re-transplanted back into the cerebella of naïve hosts to establish a propagated line for each patient-derived xenograft. Molecular classification of human tumors was previously described[145,236,237]

5.2.9 Intracranial transplantation

SCID-beige mice (6–8 weeks old) were anesthetized with Avertin (0.015mg/kg, SBMRI) and placed in a stereotaxic apparatus (Kopf, Tujunga, CA). After exposing the skull with a scalpel, a 1 mm diameter hole was drilled in the skull over the cerebellum using an 18G needle. A cell suspension (1×10^6 cells in 5-µl NB/NS-21) was slowly injected into the cerebellum at a depth of 1.5-2mm, using a 5-µl Hamilton Syringe with an unbevelled 24G needle (Hamilton, Reno, NV). After injection, the incision was closed using Vetbond.

5.2.10 *In vivo* genetic deletion and antagonist treatment

5.2.10.1 Genetic deletion

To assess the effects of survivin loss on tumor formation, tumors from SMEP mice were infected with Cre-IRES-GFP or GFP expressing retroviruses 24-36hrs and GFP+ cells were selected by sorting on a FACSVantage SE Diva or FACSAria flow cytometer (BD Biosciences). GFP+ cells were transplanted as outlined above. Alternatively, SMEP tumor cells were treated *in vitro* with 1µM 4-OH tamoxifen for 24hours, transplanted into NSG hosts, and monitored for survival. To mediate deletion in tumors *in vivo*, mice transplanted with SMEP tumors were treated with either 4.0mg tamoxifen in 200µl corn oil or corn oil control by oral gavage 2 weeks after surgery to mediate deletion of survivin in tumors.

5.2.10.2 Antagonist treatment

For systemic treatments, tumor-bearing NSGs (4 weeks post-transplant) were treated with 5mg/kg/day YM155 via microosmotic pump (Alzet, model D2004),

15mg/kg/day S12, administered 5x a week by i.p, or vehicle controls and monitored for survival.

5.2.11 Pharmacokinetic Assays

In vitro BBB-PAMPA and *in vivo* pharmacokinetic assays were performed in collaboration with the Exploratory Pharmacology core at Burnham in Lake Nona, FL. For *in vitro* assay, compounds were sent to core for the assay. Briefly, compounds are placed in the top of a microtiter plate that is separated from the bottom compartment by an artificial lipid-infused membrane immobilized on a filter. Following the permeation period, the concentration of drug in the donor and acceptor compartments is measured using UV spectroscopy. For *in vivo* evaluation of S12 distribution, tumor-bearing NSGs were treated with 15mg/kg S12 and plasma, normal brain, and tumor tissue were collected at 30 mins or 2 hours post treatment. Samples were flash frozen and sent to Lake Nona for LC/MS analysis to measure S12 levels in these tissues.

5.2.12 Flank tumor implantation and *in vivo* antagonist treatment

Cells isolated from tumors were re-suspended 1:1 in NB/NS-21 media and GFR-matrigel. 100µl of cell suspension (6-7 x 10^6 cells) was injected subcutaneously into the flanks of 5-8 week old CD-1 Nu/Nu mice. Tumor growth was monitored using calipers and tumor volume calculated using the formula 0.52*length*$width^2$. Treatment was initiated when tumors reached ~100 mm^3. For intratumoral injections, tumors were injected twice a week with YM155 (20 µM final concentration) or vehicle (20% DMSO in saline). For systemic treatments, mice were treated with 10 mg/kg/day YM155 or saline by subcutaneous micro-osmotic pump (Alzet, model D2004). Experimental treatments

were continued until control tumors reached maximum size of 2000mm^3, at which point tumors were collected for analysis.

5.2.13 Statistics

Unless otherwise indicated, statistics were calculated by ANOVA with post hoc student's t-test. p values of less than 0.05 were considered significant and marked with asterisks where appropriate.

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